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Gas and Indole Variants of *Escherichia coli* at Elevated Incubation Temperature

by

D. H. Bueschkens

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Gas and Indole Variants of *Escherichia coli* at Elevated Incubation Temperature submitted by D. H. Bueschkens in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in MICROBIOLOGY.

Dedication

This thesis is dedicated to DR. MICHAEL E. STILES, who created the basis of this experiment, and to HANS and DANICA BUESCHKENS, who created the basis of the experimenter.

Abstract

The effect of various treatments on the ability of Enterobacteriaceae to produce indole from tryptophan, and acid and gas from lactose was studied. No variants were obtained for these characteristics at 35.0°C, while at 44.5°C variants were obtained for two strains of *E. coli*. All of the *E. coli* variants failed to produce gas in EC or lactose broth at 44.5°C, using the conventional Durham tube method, but only 3 variants failed to produce indole from tryptophan at 44.5°C. The *E. coli* stock cultures from which these variants were obtained normally lost these functions at 47.0 and 48.0°C. The maximum growth temperature for both *E. coli* stock and variant cultures in TSB, EC, and lactose broths was 48.0°C. Loss of the characteristics by the variants could not be attributed to a change in growth range.

No variants were obtained for the *E. coli* cultures with repeated transfer or prolonged storage of the stock cultures on nutrient agar slants at 4°C, nor as a result of daily sublethal heat treatment at 52°C for 15 min. in Tryptic Soy Broth (TSB). A few variants were found as a result of repeated UV treatment (5 min. daily germicidal UV irradiation in TSB), extended storage at 4, 35, and 45°C in TSB, and cold storage at -16°C. In contrast, variants were found repeatedly when stored on nutrient deficient broth and semisolid agar media at 4°C and -16°C. The variants were shown to be stable upon subculture, suggesting that the

treatments generated permanent DNA alterations.

E. coli variants which did not produce gas in lactose or EC broth at 44.5°C (Durham tube method) were attributed to a more temperature-sensitive formic hydrogen lyase enzyme. This enzyme produced traces of H_2 and CO_2 gas from lactose at 44.5°C (detected by gas chromatography), about 1/10th to 1/30th of the amount produced at 44.0°C. The generation of temporary *E. coli* ATCC 11775 variants for gas production in lactose and EC broth at 44.5°C by acridine orange treatment, used to rid the *E. coli* of non-integrated F plasmids, suggested that the *lac*$^+$ genes of these variants may have been associated with their F plasmid and lost during treatment. *E. coli* variants which failed to produce indole at 44.5°C had a more temperature-sensitive synthesis and activity of tryptophanase. The exact location of the genetic alteration responsible for this variation was not determined.

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List of Symbols and Abbreviations

a	production of acid
EC	EC broth
g	gas
Glu	fermentation of glucose
Ind	production of indole
Lac	fermentation of lactose
Lac(g)$^-$	lack of gas production from lactose
MR-VP	methyl red - Voges-Proskauer medium
NA	nutrient agar
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
Suc	fermentation of sucrose
TBA	tryptone bile agar
TSB	tryptic soy broth
TSI	triple sugar iron agar
var	variant
VP	Voges-Proskauer reaction
VRBA	violet red bile agar

I. Introduction

Escherichia coli has been used for many years as a standard indicator organism for fecal contamination of water (Barrow, 1977), and of unsanitary handling or post-processing contamination of heat-processed or cooked foods (ICMSF, 1978). A proposed definition of fecal *E. coli* is a microorganism that is:

> "capable of fermenting lactose with the production of acid and gas at 44.5°C within 48 h, which does not produce cytochrome oxidase, which produces indole from tryptophane, which is incapable of producing acetyl methyl carbinol, which is incapable of hydrolysing urea and which gives a positive result with the methyl red test" (Dufour, 1977).

This definition fits the Anderson and Baird-Parker (1975) description of *E. coli* biotype I.

Public Health authorities in their attempts to identify fecal *E. coli*, have focused primarily on the ability of *E. coli* to produce acid and gas from lactose at elevated temperature. However, in recent years, the ability of fecal *E. coli* to produce indole from tryptophan at elevated temperatures has become a criterion of increasing importance. Some differences in methodology have developed between workers in Europe and North America. In Europe, the elevated incubation temperature is 44.0 ± 0.1°C, while in North America these temperatures range from 44.5 to 45.5 ± 0.2°C (ICMSF, 1978). Differences also centre on the

relative importance of the lactose and indole traits (Anderson and Baird-Parker, 1975). Approximately 90% of *E. coli* strains produce acid from lactose at 37°C within 48 h, while 99% produce indole from tryptophan under the same conditions of incubation (Ewing, Davis, and Martin, 1972).

Lactose-positive anaerogenic *E. coli* (Mossel, Visser, and Cornellisen, 1963), slow lactose-fermenters (Mehlman, Simon, Sanders, and Olsen, 1974; Mossel and Vega, 1973), and mutants for indole production and lactose fermentation (either slow producers or complete non-producers) (Anderson and Baird-Parker, 1975) would be missed by normal testing procedures. Outbreaks of gastroenteritis, such as the 1971 outbreak attributed to anaerogenic enteropathogenic lactose-negative *E. coli* in soft ripened cheese, indicated that procedures for detecting *E. coli* in foods should be re-evaluated (Fantasia, Mestrandrea, Schroeder, and Yager, 1975). Despite this, the production of gas from lactose at elevated temperature remains the most widespread test for fecal *E. coli* in food and water (Leclerc, Mossel, Trinel, and Gavini, 1977).

The production of indole from tryptophan at elevated temperatures (44.0 to 45.5°C) is becoming an accepted standard test for detection of fecal *E. coli* (Anderson and Baird-Parker, 1975; Rayman, Jarvis, Davidson, Long, Allen, Tong, Dodsworth, McLaughlin, Greenburg, Shaw, Beckers, Qvist, Nottingham, and Stewart, 1979). However, it has not yet assumed the same importance as the elevated temperature

test for gas production from lactose (ICMSF, 1978). Testing samples of various Enterobacteriaceae cultures for indole production after 24 h incubation at 44.0°C revealed that 95% of the Ind^+ organisms were *E. coli* biotype I, and only 3.4% were other fecal coliforms (Anderson and Baird-Parker, 1975). Therefore, indole production at elevated temperatures is an effective and reliable test for detecting fecal *E. coli* (Anderson and Baird-Parker, 1975).

Another concern is that environmental conditions in food processing plants may induce either temporary or permanent variations in the biochemical reactions exhibited by *E. coli*, making their detection more difficult (Ingram, 1977). Sanitizers (Maxcy, Tiwari, and Soprey, 1971; Scheusner, Busta, and Speck, 1971), acids (Przybylski and Wutter, 1979), salts (Anderson, Rhodes, and Kator, 1979), organic solvents and food additives (Ingram, 1977), desiccation (Asada, Takano, and Shibasaki, 1979; Webb, 1967), UV light (Witkin, 1976), heat treatment (Grau, 1978; Kasweck and Fliermans, 1978), freeze-drying (Tanaka, Pierson, and Ordal, 1979), and cold storage (Davenport, Sparrow, and Gordon, 1976; Ordal, 1970; Speck and Ray, 1977) have all been shown to be capable of inducing biochemical variations in *E. coli*. Of these factors, heat treatment and cold storage are most commonly used in the food industry (ICMSF, 1978), and would thus have the greatest opportunity to induce variation.

The reliability of fecal *E. coli* detection has serious implications for the safety of water and processed foods. At present this depends on the reliability of gas production from lactose at elevated temperatures of 44.0 to 45.5°C, by fecal *E. coli*. The objective of this study was to investigate the effect of mild heat treatment and storage under debilitating conditions, such as refrigeration and poor nutrition, on strains of *E. coli* biotype I, focusing on their ability to produce gas from lactose, and indole from tryptophan, at 35.0 and 44.5°C. For comparison, other Enterobacteriaceae - including *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Salmonella* species - were also tested. The effect of germicidal UV treatments was also investigated as a reference for the study.

II. Review of Literature

This review of the literature focuses on the fermentation of lactose and production of indole by *E. coli* and other Enterobacteriaceae at elevated incubation temperature. In addition, temperature and storage factors that have been shown to influence these characteristics are reviewed, including UV treatment as a reference for genetic damage and acridine orange as a reference for plasmid-related effects.

A. Lactose Fermentation and Indole Production by Enterobacteriaceae

Members of the family Enterobacteriaceae can be differentiated on the basis of their ability to produce acid and gas from lactose, and indole from tryptophan (Buchanan and Gibbons, 1974). Lactose is not fermented by *Edwardsiella*, *Hafnia*, *Serratia*, *Proteus*, and *Yersinia* species (Buchanan and Gibbons, 1974). Some species or strains of *Salmonella* and *Shigella* may produce acid from lactose at 37.0°C, but this is not normally the case (Buchanan and Gibbons, 1974). Most *Escherichia* (Ewing *et al.*, 1972), *Citrobacter* (Lund, Matsen, and Blazevic, 1974), *Enterobacter* (Ewing and Fife, 1972) with the exception of *E. hafniae* (Ewing and Fife, 1968) and *Klebsiella* with the exception of *K. ozaenae* (Cowan, Steel, Shaw, and Duguid, 1960) ferment lactose at 37.0°C to produce both acid and gas. At elevated temperatures between 44.0 to 45.5°C, some

strains of *Citrobacter* and *Enterobacter* may still produce acid but not gas from lactose, while many strains of *E. coli*, *K. pneumoniae* (Bagley and Seidler, 1977) and *K. oxytoca* (Naemura and Seidler, 1978) are capable of producing both acid and gas from lactose.

These divisions are not always so clear cut. In addition to normal strain variations, mutations for lactose fermentation may also occur (Buchanan and Gibbons, 1974). For example, some species of *Salmonella* which do not normally produce acid from lactose at 37.0°C (Ewing, 1972; Harvey and Price, 1979) have generated variants which are capable of producing acid from lactose (Anand, Finlayson, Garson, and Larson, 1980; Blackburne and Ellis, 1973; Gonzalez, 1966; Le Minor, Coynault, and Pesson, 1974). *E. coli* variants which do not produce gas from lactose at 44.5°C, or which have lost the ability to ferment lactose at all, have also been reported (Kanai, Uchino, Sakeniwa, Sumino, and Miyazawa, 1975; Kasweck and Fliermans, 1978; Rychert and Stephenson, 1981).

Some researchers claimed that environmental factors may influence the ability of microorganisms to ferment lactose and other sugars (Blackburne and Ellis, 1973). Others have claimed that the *lac*$^+$ genes may be transmitted by plasmids (Le Minor *et al.*, 1974). These plasmids have been derived from *E. coli* or other Lac$^+$ Enterobacteriaceae (Anand *et al.*, 1980). Loss or gain of ability to ferment lactose would thus be dependent upon the loss or gain of the plasmid bearing

the *lac*$^+$ gene or of the gene itself (Anand *et al.*, 1980). Under laboratory conditions it has been demonstrated that plasmid transfers occur among different genera of the Enterobacteriaceae (Anand *et al.*, 1980; Baron, Gemski, Johnson, and Wohlhieker, 1968).

According to the eighth edition of Bergey's Manual (Buchanan and Gibbons, 1974) the genera *Salmonella*, *Enterobacter*, *Hafnia*, and *Serratia* do not produce indole from tryptophan at 37.0°C. Most *Escherichia* (Ewing *et al.*, 1972) and *Edwardsiella*, and some strains or species of *Citrobacter*, *Shigella*, *Proteus*, *Yersinia*, and *Klebsiella* produce indole from tryptophan at 37.0°C (Buchanan and Gibbons, 1974). At elevated temperatures such as 44.5°C, *E. coli* and *K. oxytoca* retain the ability to produce indole from tryptophan, while other organisms become more variable for this trait (Naemura and Seidler, 1978). Although the ability to produce indole may be lost, it has been reported that indole variants do not appear as often as lactose variants, and plasmid transmission of indole genes among different genera of Enterobacteriaceae has yet to be demonstrated (Anand *et al.*, 1980).

B. Lactose Fermentation

Lactose Transport by *E. coli*

Unlike other sugars, the lactose transport system in *E. coli* does not use the phosphotransferase system (Wong and MacLennan, 1973). Lactose is concentrated in the cells,

without being chemically altered (Andrews and Lin, 1976). This is mediated by the enzyme β-galactoside permease, a membrane protein known as the M protein, which binds one mole of galactoside per mole of enzyme (Wong and MacLennan, 1973). The M protein transports the bound β-galactoside across the cell membrane either by facilitated diffusion (under conditions of energy deprivation) or by active transport (under conditions of sufficient energy) (Andrews and Lin, 1976). The β-galactoside is released by the M protein inside the cell, and hydrolysed to glucose and galactose by β-galactosidase (Wong and MacLennan, 1973).

Lactose transport in *E. coli* can be blocked by inhibitors of electron transport or by mutations which inhibit transport (Wong and MacLennan, 1973). Total carriers for lactose can be determined independently of energy-coupled carriers for lactose. ONPG tests can be used to detect functional carriers for lactose, while thio-methyl-galactoside tests can be used to detect energy-coupled carriers for lactose. For an *E. coli* to be ONPG$^+$ it must be able to transport lactose into the cell (Wong *et al.*, 1970) and to hydrolyze it to glucose and galactose (Rychert and Stephenson, 1981). It would therefore appear that any severe damage to the lactose transport system of *E. coli* - i.e. either a non-functional β-galactoside permease or a non-functional β-galactosidase system - could be detected by a negative ONPG test.

More extensive testing would be required to determine the exact location of such damage within the lactose transport system. Therefore, partial (i.e. energy-coupled) and complete (i.e. total) lactose carrier mutants can be distinguished (Wong, Kashket, and Wilson, 1970). Partial carrier mutants for lactose could still transport lactose into the cell by facilitated diffusion, although the lower amount of lactose entering the cell may make it more difficult to detect lactose fermentation by-products such as acid and gas. Complete carrier mutants for lactose would be unable to transport lactose into the cell at all, totally preventing lactose fermentation.

Lactose transport in *E. coli* can be blocked by mutations in β-galactoside permease (the M protein) or β-galactosidase (Andrews and Lin, 1976). Increasing the incubation temperature from 35.0 to 44.5°C, reduces the rate of lactose and glucose uptake in nonfecal coliforms (because of their temperature-sensitive transport system), but not in fecal coliforms (Dockins and McFeters, 1978). However, temperature-sensitive mutants with mutations in the *lac*Y permease gene in *E. coli* have been isolated which synthesize normal levels of β-galactoside permease at 25°C, but greatly decreased levels at 42°C (Crandall and Koch, 1971). The β-galactoside permease produced by these mutants is not temperature-sensitive, but the rate of synthesis of the functional permease is temperature-sensitive, causing lactose uptake to be decreased at 42°C as it is in nonfecal

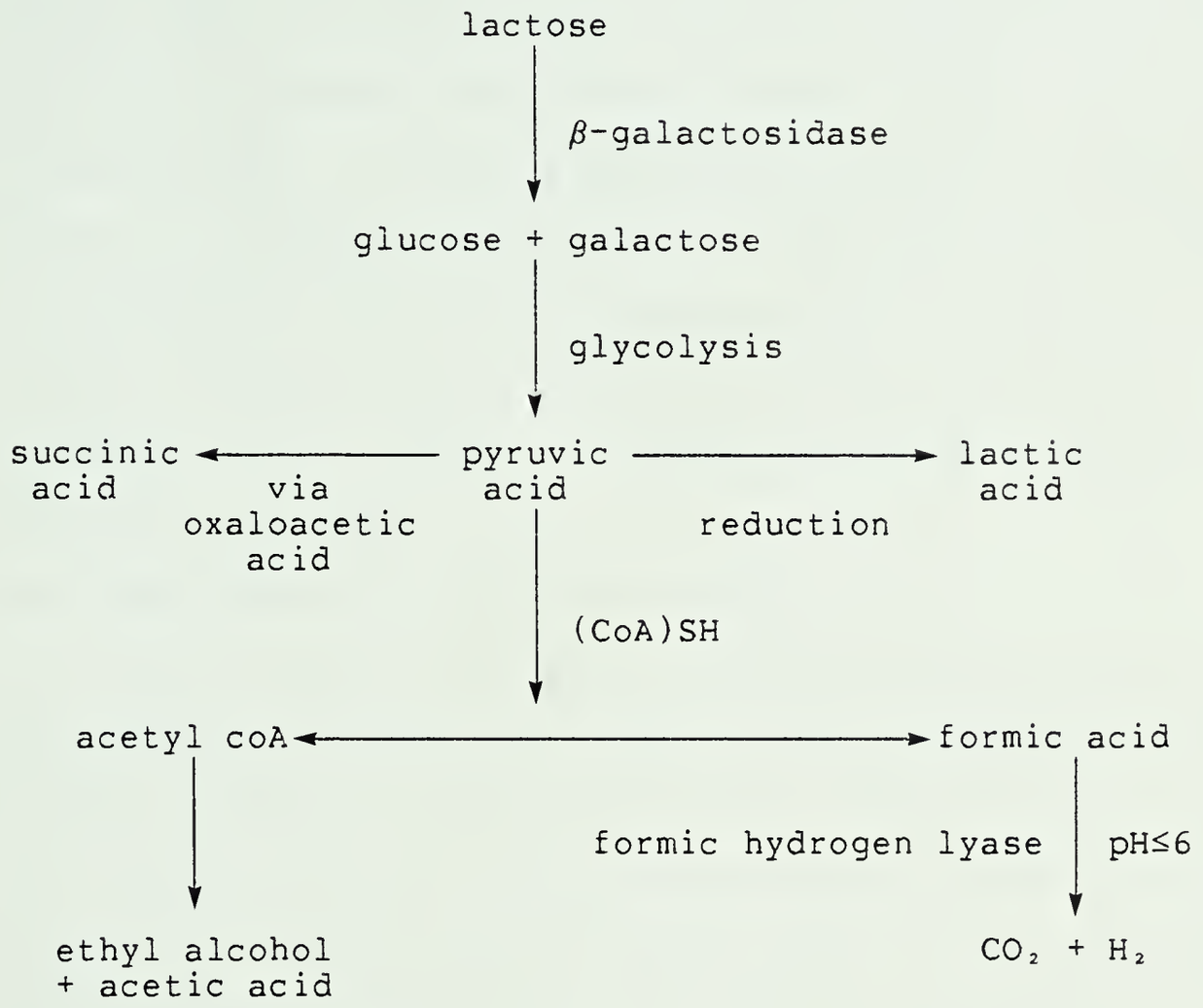
coliform bacteria (Crandall and Koch, 1971).

Mutants with a defective plasma membrane-cell wall complex that permit the loss of β-galactosidase from the cell, can grow in lactose even if they are unable to transport β-galactosides (Olden and Wilson, 1972). At 44.5°C, the β-galactosidase of nonfecal coliforms is thermally inactivated, but not in the case of fecal coliforms (Dockins and McFeters, 1978). However, *E. coli* mutants with a temperature-sensitive (43°C) Z region of the *lac* operon (Simon and Apirion, 1972) or a pH sensitive Z region (Colb and Shapiro, 1977) have been reported which are blocked at the level of β-galactosidase enzyme synthesis and cannot metabolize lactose, mimicing the behavior of nonfecal coliforms under these conditions. A temperature-sensitive (44.5°C) *E. coli* mutant which is $ONPG^-$ EC^- $Lac(g)^-$ at 44.5°C and possesses only half of the normal β-galactoside permease activity has also been isolated (Rychert and Stephenson, 1981).

The Lactose Fermentation Pathway for *E. coli*

Lactose fermentation in *E. coli* generally follows the pathway illustrated in Figure 1. The amount of each end product depends on the strain of *E. coli* and the conditions under which it is grown and tested (Porter, 1947). The major end products tend to be the same as those for glucose fermentation: ethanol; formic, acetic, and lactic acids; with lesser amounts of succinic acid and traces of other end-products (Blackwood, Neish and Ledingham, 1956). At

FIGURE 1. Significant Reactions of Lactose Fermentation for Gas Production by *E. coli*.



(Davis, Dulbecco, Eisen, Ginsberg, and Wood, 1973; DeLey, 1962; Pelczar, Reid, and Chan, 1974)

acidic pH (values around pH 6) ethanol, acetic and lactic acids, CO_2 and H_2 accumulate, while at more alkaline pH (pH 7.4 - 7.8) formic acid accumulates (Blackwood *et al.*, 1956; Porter, 1947). On the average, 2.5 moles of acid are formed per mole of lactose fermented by *E. coli* (Stokes, 1949). As the end products of lactose and glucose fermentation are similar, an example of the amount of each end product formed can be shown by the following data reported by Stokes (1949): 0.8 moles ethanol, 1.2 moles formic acid (or its equivalent in CO_2 and H_2), 0.2 moles lactic acid, and 0.4 moles succinic acid.

Gas Production from Lactose by *E. coli*

The most important aspect of lactose fermentation for the standard detection of *E. coli*, is the production of gas by the breakdown of formic acid to CO_2 and H_2 (Davis *et al.*, 1973). Gas production from lactose at 44.0 to 45.5°C is the primary test used to detect fecal *E. coli* in food and water sanitation tests (Leclerc *et al.*, 1977). This reaction is most specific for the identification of *E. coli* at 45.5°C, but more false negatives occur at 45.5°C than at 44.0°C (Fishbein and Surkiewicz, 1964). Debate persists on which temperature is best for standard detection of fecal *E. coli* (Anderson and Baird-Parker, 1975; Dufour, 1977).

The enzyme formic hydrogen lyase is responsible for the conversion of formic acid to H_2 and CO_2 gas (Davis *et al.*, 1973). It is most active at acidic pH (about pH 6) and least active at neutral to alkaline pH (Bovarnick, 1965). At

elevated temperatures the activity of formic hydrogen lyase is reduced (Leclerc *et al.*, 1977). Inhibition is greater as incubation temperature is increased, but the inhibition is normally reversible (Fishbein, 1962). At 45.5°C, approximately 92% of *E. coli* strains produce gas from lactose, while at 46.5°C only 64.5% produce gas (Fishbein, 1962).

Factors which inhibit the formation of formic acid by *E. coli* also inhibit gas production from lactose, even if a functional formic hydrogen lyase enzyme is present (Kanai *et al.*, 1975). Atypical *E. coli* biotype I strains have been discovered which lack the ability to produce gas in EC or lactose broth at 44.5°C, but can produce gas in broths containing formic acid at 44.5°C (Kanai *et al.*, 1975). Kanai *et al.* (1975) found that these strains produce a functional formic hydrogen lyase enzyme, but fail to produce gas from lactose, due to an inhibition earlier in the lactose fermentation pathway, presumably in the conversion of pyruvic to formic acid.

The amount of gas produced by *E. coli* can vary greatly, depending upon the strain, the conditions to which it is exposed, and any environmental damage it may have sustained (Meadows, Anderson, Patel, and Mullins, 1980; Mossel and Vega, 1973). The inclusion of buffering agents in media used to detect gas production decreases the variability among subcultures, and increases gas production by preventing rapid pH change which might affect cell metabolism (Meadows

et al., 1980b). Components of selective media (for example, brilliant green bile and sodium ricinoleate) inhibit or reduce gas production by reducing the amount of *E. coli* growth (Meadows, Anderson, and Patel, 1980). Elevated incubation temperatures also reduce gas production by reducing the amount of growth of the organism (Meadows *et al.*, 1980a). Other stressful environmental conditions which affect the amount of growth also reduce gas production (Anderson, Meadows, Mullins, and Patel, 1980). Reduced gas production could be important because it might lead to false negative tests for fecal *E. coli* (Anderson *et al.*, 1980).

The relative amounts of H_2 and CO_2 gas produced by *E. coli* can vary greatly (DeLey, 1962). Under ideal conditions, the H_2:CO_2 ratio approaches unity, but there is generally a slight excess of CO_2 compared to H_2 (Keyes and Gillespie, 1913). The presence of oxygen, nitrites and sodium phosphate increase the amount of CO_2 relative to H_2 produced (Keyes and Gillespie, 1913). Researchers have reported finding H_2:CO_2 ratios from 1:1 to 1:7.7 (DeLey, 1962), 1:0.32 to 1:2.18 (Keyes, 1909), 1:1.06 to 1:5.4 (Keyes and Gillespie, 1913), and 1:1.2 to 1:6.8 (Blackwood *et al.*, 1956). However, the most frequently reported H_2:CO_2 ratio is approximately 1:1 (DeLey, 1962).

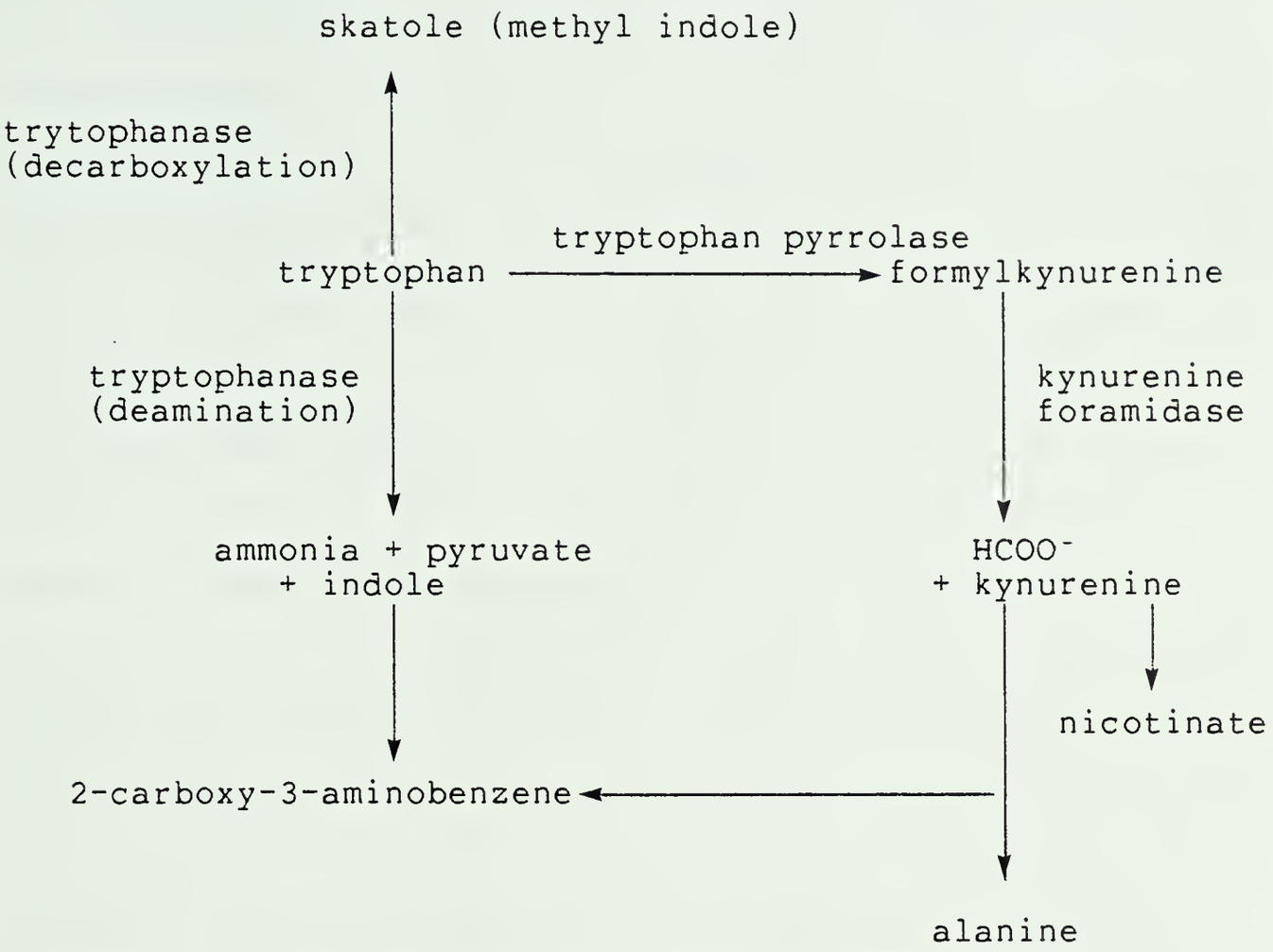
C. Indole Production by *E. coli*

The Pathway for Indole Production by *E. coli*

The major steps in the pathway used for the production of indole from tryptophan by *E. coli* is illustrated in Figure 2. Less is known about this pathway than about the lactose fermentation pathway (Lamanna and Mallette, 1965). The direct pathway from tryptophan to indole, ammonia and pyruvate is believed to be the major route for indole production (MacFaddin, 1977). Most assays for indole use the reagent *p*-dimethylaminobenzaldehyde (Anderson and Baird-Parker, 1975). This reagent, in the presence of HCl, water, and warmed condensation, combines with the intact pyrrole structure of indole, to form a red-violet colour. It may also combine with the same pyrrole group in skatole (methyl-indole) to form an orange-red colour. In the absence of a pyrrole group, a yellow colour is produced (MacFaddin, 1977).

Sugars should be kept to a minimum in the growth medium, because carbohydrates can delay indole production for up to 5 days, due to catabolic repression (Mundt and Rat, 1962). Indole variants of *E. coli* would be expected to possess some genetic alteration in their indole pathways, most probably at the level of the main enzymes - tryptophanases or tryptophan pyrrolase (Lamanna and Mallette, 1965). Although little is known about indole mutants of *E. coli*, it is known that the tryptophanase enzyme catalyzes the reversible conversion of *l*-tryptophan

FIGURE 2. Major Steps in Indole Production from Tryptophan by *E. coli*



(Lamanna and Mallette, 1965; MacFaddin, 1977).

to indole, pyruvate, and ammonia (White and Yudkin, 1979). The activity of this enzyme is catabolic, and dependent on pyridoxal pyruvate (Gartner and Riley, 1965).

Tryptophanase synthesis is induced by the presence of *l*-tryptophan or its analogue "5-methyl-DL-tryptophan" (Gartner and Riley, 1965). The enzyme is encoded by the *tna* gene, with constitutive mutants tentatively linked to a *tna*R locus of unknown nature (White and Yudkin, 1979). Regulatory mutants (not linked to the *tna* gene, but of undefined location) may enable tryptophanase synthesis to be unusually insensitive to catabolic repression. This creates the potential for indole production even in the presence of high concentrations of carbohydrates (Yudkin, 1976). Promoter mutants believed to be closely linked to the *tna* gene may also affect the rate of synthesis of tryptophanase. This has the potential to affect the amount of indole which can be produced (Ward and Yudkin, 1976). Temperature-sensitive (41°C) mutants for both tryptophanase synthesis and activity have also been isolated, and they are believed to be caused by mutations of undefined nature in the tryptophanase structural gene (Taylor and Yudkin, 1978). Further study is required to determine the exact nature of these tryptophanase mutations, but all could conceivably affect the production of indole by *E. coli* by affecting tryptophanase synthesis and activity.

D. Effect of Debilitating Treatments on *E. coli*

Various conditions may affect the biochemical reactions exhibited by *E. coli*, making the detection of the organism more difficult (Ingram, 1977). High and low temperature treatments are among the most common environmental stresses to which cells are exposed in food processing. Foods are frequently heat-processed to kill bacteria, while many foods are refrigerated or frozen to reduce spoilage and retard the growth of any spoilage or pathogenic organisms (ICMSF, 1978). Cells which survive such treatments may sustain damage to their cell membranes, RNA, and DNA, causing enzyme functions to be weakened or altered (Alur and Grecz, 1975; Patterson and Jackson, 1979). If the enzymes involved in either the production of indole from tryptophan or gas from lactose at elevated temperature are altered or weakened by such treatments, *E. coli* may become more difficult to detect by standard detection methods.

High Temperature Treatment

Heat treatment at 52°C or above for extended periods of time is usually fatal to *E. coli* (Pellon and Gomez, 1981). However, at lower temperatures (43 to 50°C) cells may be injured instead of killed, provided that the length of heat exposure is not excessive (Grau, 1978). Injured cells re-inoculated into a suitable recovery medium may recover their normal characteristics after an extended lag time (Clark and Ordal, 1969; Stiles and Witter, 1965; Tomlins, Yoh, Takeda, and Miwatant, 1971). Depending on the length of

resuscitation time allowed for a heat-treated culture to recover in a nonselective medium before placing it into a selective medium, the heat injury may not be repaired (killing the cell), may be partially repaired (impairing certain cellular functions), or it may be totally repaired (no change detected) (Pellon and Gomez, 1981). For instance, 5% of the surviving *E. coli* heated at 50°C for 30 minutes were recovered in Luria broth media without resuscitation, while 50% were recovered when the heated cells were resuscitated for 3 h in the defined growth medium (Pellon and Gomez, 1981). In general, the longer the resuscitation time, the greater the possibility that damage will be repaired (Pellon and Gomez, 1981).

Sublethal heat treatment is often more destructive than freezing (Ordal, 1970). It exerts its effect at the level of the cell membrane, RNA, and DNA (Ordal, 1970; Tomlins *et al.*, 1971). Single and double-stranded breaks in DNA or even breaks in the folded chromosome of *E. coli* can be generated by temperatures of 50 to 60°C (Pellon, Ulmer, and Gomez, 1980), with the level of recovery dependent on the effectiveness of the DNA repair system (Sedgwick and Bridges, 1972). Damage to the cell membrane decreases viability due to the loss of K^+, Mg^{++}, Ca^{++} and amino acids, and by decreasing the accumulation of lactose and other sugars within the cell (Grau, 1978). Survival of *E. coli* during sublethal heat treatment can be increased if a high concentration of soluble carbohydrates is present, if the

levels of NaCl, Mn^{++}, and Co^{++} are low, and if the length of heating is minimized (Lee and Goepfert, 1975). Degradation of ribosomal RNA and DNA may lead to lower enzyme activities, altered transport kinetics, and lipid content, and a need for more growth requirements (D'Aoust, 1978). However, some enzymes may be only moderately susceptible to heat injury, and would thus not be affected by such heat treatment (Tomlins *et al.*, 1971).

Long-term mild thermal stress has been shown to affect the biochemical reactions of *E. coli*. The discovery of *lac*$^+$ to *lac*$^-$ shifts in thermally stressed *E. coli* in reactor effluent waters is a prime example of this phenomenon (Kasweck and Fliermans, 1978). In addition, when prolonged incubation at 44.5°C was combined with enrichment in sodium lauryl sulfate, researchers noted a loss of plasmids and a less efficient recovery of *E. coli* from foods (Hill and Carlisle, 1981). Damage induced by long-term mild thermal stress should be less severe than sublethal heat treatment, as cells are exposed to a less intense level of injury (Ordal, 1970). Both types of injury should exert similar effects on the cell membrane, RNA, and DNA, but longer periods of exposure would be required to achieve the same degree of cell injury for cultures exposed to mild thermal treatment (Ordal, 1970).

Low Temperature Treatment

Temperatures below 5°C generally inhibit growth of *E. coli* and other pathogenic Enterobacteriaceae (Davenport *et*

al., 1976). Cell death is increased if the food is processed (cooked, salted, or acidified) in addition to refrigeration or freezing, although damage incurred by low temperature storage may be minimized if the food is viscous and high in nutrients (Speck and Ray, 1977). Gram-negative bacteria are more sensitive to freezing damage than Gram-positive bacteria, probably due to differences in their cell wall structure though this has not yet been proven (Calcott, 1978). Cells in the log phase of growth are more sensitive than cells in the stationary phase, because of more rapid DNA synthesis (Speck and Ray, 1977). Storage of *E. coli* at 1 and 4°C for 35 days markedly affected their growth on selective media, and caused injury similar to that sustained by more severe treatments such as freezing and sublethal heating (Patterson and Jackson, 1979). Storage at 1°C for 35 days caused only a 1 log decrease in cell survival, whereas storage at 4°C for 35 days caused a 2 to 3 log decrease in survival, indicating that cell injury was more severe at 4°C than at 1°C (Patterson and Jackson, 1979).

When storing cultures, bacteria may live for years at 4°C, but care should be taken with cultures stored at -20°C, the eutectic point of most liquid media (Lapage, Shelton, Mitchell, and Mackenzie, 1970). This is also the temperature at which the greatest amount of freeze-thaw damage occurs in cells stored in liquid media (Calcott, 1978). The extent of freeze-thaw damage depends on the freezer temperature and the cooling rate (Hanafusa, 1967). Storage of cultures at

-70°C is preferable to -20°C, because it reduces the amount of freeze-thaw damage (Lapage *et al.*, 1970). Cells cooled slowly survive better than those cooled rapidly (Calcott, 1978). A cooling rate of 6°C/min. is best for *E. coli*, though cooling at 1°C/min. is optimal for most cells (Calcott, 1978). The main cause of cell injury during rapid freezing is the formation of intracellular ice crystals (Nei, Araki, and Matsusaka, 1967). This causes the cell membrane to lose its integrity and the DNA to fragment by single-strand breaks (Alur and Grecz, 1975). Anaerobic cultures are more sensitive to this type of damage than aerobic cultures (Nei *et al.*, 1967).

Prolonged exposure to the dehydrating effects of ice crystal formation and increased solute concentrations occurring at temperatures around -20°C also decrease cell viability. Slow cooling past this point results in more cell injury than rapid cooling (Rowe, 1967). Freeze-thaw damage can thus be minimized by a slow rate of cooling to -20°C and a rapid cooling past that point (Lapage *et al.*, 1970). Similarly, the rate of thawing should be as rapid as possible to minimize the freeze-thaw damage induced by ice crystals (Lapage *et al.*, 1970).

Enzyme denaturation by freeze-thaw damage is not caused by ice crystal formation, because of the large size of ice crystals compared to the size of protein molecules (Hanafusa, 1967). Denaturation is believed to be due to the effect of salt concentration increasing with ice crystal

formation in the vicinity of the enzyme, and the partial unfolding of the helical structure of fibrous proteins upon freezing (Hanafusa, 1967). Many cellular functions are dependent on protein complexes whose structure might be sensitive to freeze-thaw damage, though the individual proteins within that complex may not be sensitive to freeze-thaw damage (Hanafusa, 1967).

Freeze-thaw damage can be further reduced by keeping electrolytes to a minimum (Lapage *et al.*, 1970), minimizing the water available for ice-crystal formation (Calcott, 1978), and excluding acids, surface active agents, and certain enzymes such as lysozyme and proteases from the storage medium (Speck and Ray, 1977). The inclusion of cryoprotective agents in the storage medium provides the cells with further protection (Lapage *et al.*, 1970). Cryoprotectants include a variety of triglycerides, proteins, and carbohydrates (Speck and Ray, 1977), the most frequently used cryoprotectants are glycerol, sugars, starches, and complex undefined agents such as blood proteins or malt extracts (Calcott, 1978). For most efficient storage, *E. coli* cells should be in the stationary phase of growth, moderate to high population density, and grown under conditions which promote the synthesis of protective compounds such as polyglucose or glycogen-like reserves (Calcott, 1978). *E. coli* in the log phase of growth, low population density, or grown in stressed or poor nutritive conditions have stressed DNA synthesis systems and

are thus more vulnerable to freeze-thaw damage (Calcott, 1978).

Cells that survive freeze-thaw damage are initially more sensitive than normal cells to osmotic change, incubation conditions, and media composition (Calcott, 1978). However, the injured cells generally regain their normal functions upon subculture, since the damage incurred is usually not mutagenic (Calcott, 1978). In cases where resuscitation time is minimal or genetic damage is incorrectly repaired, altered enzyme functions may result (Ordal, 1970). The incidence of such alterations due to freeze-thaw damage is less frequent than alterations due to heating, because the type of DNA damage is less severe and more easily repaired (Ordal, 1970).

UV Treatment

The type of DNA damage induced by mild heat treatment may also be induced by ionizing radiation such as X-rays or gamma rays (Bridges, Ashwood-Smith, and Munsen, 1969). UV light, a non-ionizing radiation, has a more mutagenic effect on cells than either ionizing radiation or mild heat treatment (Whitkin, 1976). UV treatment is commonly used as a basis for comparison with other suspected mutagens, because the mechanism of inducing mutation by UV irradiation has been well established (Lewin, 1977). Short-wavelength germicidal UV radiation (less than 280nm) promotes photochemical reactions in nucleic acids, causing the production of cyclobutapyrimidines and single and

double-stranded DNA breaks due to nicks induced by direct hits on the DNA (Murphy, 1975). Intermediate solar wavelength UV radiation (280 to 315nm) may induce similar damage, but is not normally as severe (Murphy, 1975). UV damage is normally repaired by the cell's excision-repair and recombination repair systems (Rupp, Wilde, Reno, and Howard-Flanders, 1971). When such repair systems are defective or fail to repair a dimer correctly, some cell functions may be either temporarily or permanently lost or altered (Whitkin, 1976). This may result in the loss or alteration of an enzyme synthesized by the cell (Whitkin, 1976).

Acridine Orange Treatment

Acridine orange (3,6-bisdimethylaminoacridine hydrochloride) is a potent mutagen and plasmid-curing agent (Albert, 1966). It is used more often than other plasmid-curing agents (e.g. acriflavine, proflavine, or acridine yellow) because of its low toxicity for cells (Hirota, 1960). It is often used as a standard to test if the F plasmid in a particular *E. coli* is autonomous and susceptible to curing, or integrated and cannot be cured (Lewin, 1977). Loss of non-integrated F plasmids is irreversible, as would be expected for the loss of genetic information (Hirota, Fujii, and Nishimura, 1966).

The damage caused by acridine orange is believed to inhibit the initiation of F plasmid replication (Lewin, 1977). For example, Hirota *et al.* (1966) demonstrated that

an *E. coli* culture treated with 20 ug acridine orange lost 96.2% of its F plasmids and 0.4% of its R plasmids. The main factors affecting the rate of F^+ to F^- conversion are the concentration of acridine ions and the growth of cells in the presence of the dye (Hirota, 1960). Most *E. coli* cells grown in the presence of 20 to 100 ug acridine orange per ml, for 6 or more hours at approximately 37°C will be cured of non-integrated F plasmids (Hirota, 1960). Such treatment can be used to test for a non-integrated F plasmid in an *E. coli* culture and any functions associated with that plasmid (Lewin, 1977).

III. Methods and Materials

A. Bacterial Cultures

Freeze-dried cultures from the American Type Culture Collection were used in this study, including: *Citrobacter freundii* ATCC 8090; *Enterobacter aerogenes* ATCC 13047; *Enterobacter cloacae* ATCC 13048; *Enterobacter hafniae* ATCC 13337; *Escherichia coli* ATCC 11775; *Klebsiella pneumoniae* ATCC 13883; *Salmonella choleraesuis* ATCC 13312; and *Salmonella typhimurium* ATCC 13311. In addition, laboratory cultures of the following isolates obtained from meat and stored on nutrient agar slants were used: *Enterobacter agglomerans*; and *Escherichia coli* #1840 (Stiles and Ng, 1980).

B. Maintenance of Stock Cultures

Stock cultures were maintained and routinely checked, to ensure the stability of those biochemical characteristics examined, under normal conditions of laboratory storage. For the first two months of the experiment, the stock cultures were subcultured weekly on NA plates and slants, incubated at 35.0°C for 24 h, and stored at 4.0°C. Confirmation of an organism's identity and purity was checked weekly. Culture purity was verified microscopically by Gram staining, and by streaking onto NA. The biochemical characteristics of these strains were checked weekly at 35.0°C using the BBL Minitek enteric system. The following commercially prepared discs

were used to determine the biochemical reactions of the organisms at 35.0°C: nitrate, phenylalanine, hydrogen sulfide, Voges-Proskauer, citrate, ONPG, urea, lysine, arginine, ornithine, dextrose, malonate, adonitol, arabinose, inositol, raffinose, sorbitol, lactose, rhamnose, and sucrose (BBL Minitek, 1979).

After the initial two month period, all stock cultures were subcultured monthly onto NA plates and slants. Gram staining and Minitek tests were also conducted each month to check the stability of each culture. All stock cultures were stored at 4.0°C, until required for use. The stock cultures were also inoculated into tubes of TSI, TSB, EC and lactose broth each week, and incubated at 35.0 and 44.5°C for 48 h, to check the stability of the H_2S, indole, EC, glucose, lactose, and sucrose reactions. These tests were done weekly for a period of 300 days.

C. Treatments for Obtaining Variants

Prolonged Storage of Stock Cultures

NA slants of *E. coli* ATCC 11775 and isolate #1840 from meat were stored at 4.0°C without subculturing for 166 days. These cultures were sampled and tested twice a month, in the same manner as the other stock cultures.

Heat Treatment

The stock cultures of *E. coli* ATCC 11775 and isolate #1840 from meat, *E. agglomerans*, *E. hafniae* ATCC 13337, *K. pneumoniae* ATCC 13883, and *C. freundii* ATCC 8090 were

prepared for use by inoculating sterile 9ml TSB tubes, and incubating at 35.0°C for 24 h. The tubes were subcultured daily for one week, before use in the heat treatment study. The following heat treatment, modified from Stiles, Roth, and Clegg (1973), was conducted on these cultures for 124 successive days.

The TSB cultures of the test organisms were inoculated into 200ml of sterile TSB in a 500ml Erlenmeyer flask tempered to 35.0°C, to give an OD_{600} value of approximately 0.1. The inoculated TSB was incubated at 35.0°C until an OD_{600} of approximately 1.0 had been reached. A 1.0ml aliquot of these TSB cultures was added to 99.0ml of sterile TSB in a 250ml Erlenmeyer flask tempered to 52.0°C in a temperature-controlled shaker incubator. Timing for the heat treatment was commenced 30 seconds after inoculation, to allow temperature equilibration. The cultures were heated for 15 minutes, then placed in an ice bath for 1 minute to reduce the temperature to approximately 23°C (room temperature). The cooled cultures were then incubated at 35.0°C for 20 h, for similar heat treatment the next day. Once a week, 1.0ml samples of the unheated and heated (52.0°C, 15 min.) cultures were tested for culture variants, according to the technique outlined in Section E.

UV Treatment

The same stock cultures used for the heat treatment study were prepared in the same manner for UV treatment. After 24 h incubation at 35,0°C, a 1.0ml aliquot of each

culture was placed in a sterile Petri dish and irradiated for 5 minutes with a broadbeam germicidal UV lamp (Canlab, UV Products line). A 0.1ml aliquot of each irradiated and non-irradiated culture was used for the daily TSB subculture. The remaining amount of each culture was placed in 9ml 0.1% (w/v) peptone water blanks, and examined for variants as described in Section E. This daily subculturing was conducted for the 93 day duration of this experiment. Cultures were tested for variants on a daily basis for 2 months, then on a weekly basis for the duration of the test period.

Extended Storage Treatment

Stock cultures of *E. coli* ATCC 11775 and isolate #1840 were used to inoculate each of three 500ml Erlenmeyer flasks containing 100ml sterile TSB. These cultures were incubated at 35.0°C for 24 h. One flask of each culture was stored at 4.0, 35.0, and 45.0°C, and sampled daily until the cultures had died or after 100 and 75 days at 4.0 and 35.0°C, respectively, when the experiment was terminated because the culture had been used up. Daily, 0.1ml samples were serially diluted in 9ml 0.1% (w/v) peptone water blanks at 0°C, and examined for variants as described in Section E.

Cold Storage Treatment

The same stock cultures as those used for the heat treatment, and in addition *S. typhimurium* ATCC 13311, were inoculated into 250ml Erlenmeyer flasks containing 100ml of TSB and incubated at 35.0°C for 24 h. These TSB cultures

were dispensed in 2.5ml aliquots into sterile glass vials, and stored in a freezer at -16°C. The vials of each culture were sampled immediately before freezing, after 24 h storage, and then on a weekly basis. Prior to sampling, the cultures were thawed in a 23°C water bath for 3 minutes. The samples were serially diluted in 9ml 0.1% (w/v) peptone water blanks, and examined for variants according to the method described in Section E.

Sloppy Agar Cultures

Sloppy agar was prepared according to the following formula: 3.0g trypticase, 7.5g agar, and 1 liter distilled water (Ng, Personal Communication). It was dispensed in 2.5ml aliquots into sterile glass vials, sterilized, and sealed. The same stock cultures as those used for the heat treatment study were streaked onto NA plates, and incubated at 35.0°C for 24 h. An isolated colony for each culture was inoculated into a sloppy agar vial. The vial cultures were incubated at 35.0°C for 24 h, before storage. Half the vials were stored in a refrigerator at 4.0°C, and the remainder in a freezer at -16°C.

Cultures stored at -16°C were thawed in a 23°C waterbath for 3 minutes prior to use. One vial for each culture was sampled immediately prior to storage, after 24 h storage, and once a week, thereafter. Sampling was conducted over a period of 142 days. Samples were serially diluted with 9ml 0.1% (w/v) peptone water blanks, and examined for variants by the technique described in Section E.

Nutritional Treatments

The standard composition of TSB (Difco) is as follows: 17.0g tryptone, 3.0g soytone, 2.5g dextrose, 5.0g NaCl, 2.5g dipotassium phosphate, and 1 liter distilled water. Due to the difference in nutritional level between this and the sloppy agar treatment medium, and between the number of variants obtained for those two media in this study, a range of nutritional broths and semisold media was prepared to give different levels of nutrient media. The nutritional broths and semisolid agars prepared for use in this study are listed in Table 1. Both were dispensed in 2.5ml amounts into sterile glass vials.

The stock cultures of *E. coli* ATCC 11775 and #1840 were inoculated onto NA plates, and incubated at 35.0°C for 24 h. Colonies of each culture were used to inoculate vials containing the nutritional study broths and semisolid agars, and incubated at 35.0°C for 24 h. The semisolid agars were stored at 4.0°C, while the broths were stored at -16°C. The cultures were sampled immediately prior to storage, after 24 h of storage, every 2 days for the first 2 weeks, and on a weekly basis thereafter. The frozen cultures were thawed in a 23°C waterbath for 3 minutes. The cultures were serially diluted in sterile 9ml 0.1% (w/v) peptone water blanks, and examined for variants by the technique described in Section E.

TABLE 1. List and composition of nutritional broths and semisolid agars used to study nutritional effects on production of variants by *E. coli* ATCC 11775 and isolate #1840.

Nutritional Broths and Semisolid Agars

TSB[1] 30g/L (full strength)-complete*

- without soytone*
- without dextrose*
- without soytone and dextrose*
- without salts (NaCl and dipotassium phosphate)

TSB 20g/L - complete

TSB 15g/L* - without soytone
- without dextrose
- without soytone and dextrose*
- without salts

TSB 10g/L - complete

TSB 3g/L - complete*
- without soytone
- without dextrose
- without soytone and dextrose*
- without salts

tryptone[2] 3g/L

tryptone 10g/L

tryptone 20g/L

tryptone 30g/L

*used as a semisolid agar containing 7.5g agar/l, and as a broth
[1]TSB = Tryptic Soy Broth (Difco)
[2]Same as conventional sloppy agar

D. Plating Media for Screening Variants

The three plating media used for screening variants included: nutrient agar (Difco), violet red bile agar (Difco), and tryptone bile agar (Anderson and Baird-Parker, 1975). Between 30 and 150 cells were inoculated per plate to ensure sufficient colony growth without overcrowding.

Nutrient agar (NA) was used as a general growth medium, to recover injured cells which were unable to grow well on the two selective media. Colonies from NA plates were selected for further study on the basis of size. Injured cells were likely to form smaller colonies than normal. Colonies were also selected at random if no morphologically different colonies were detected.

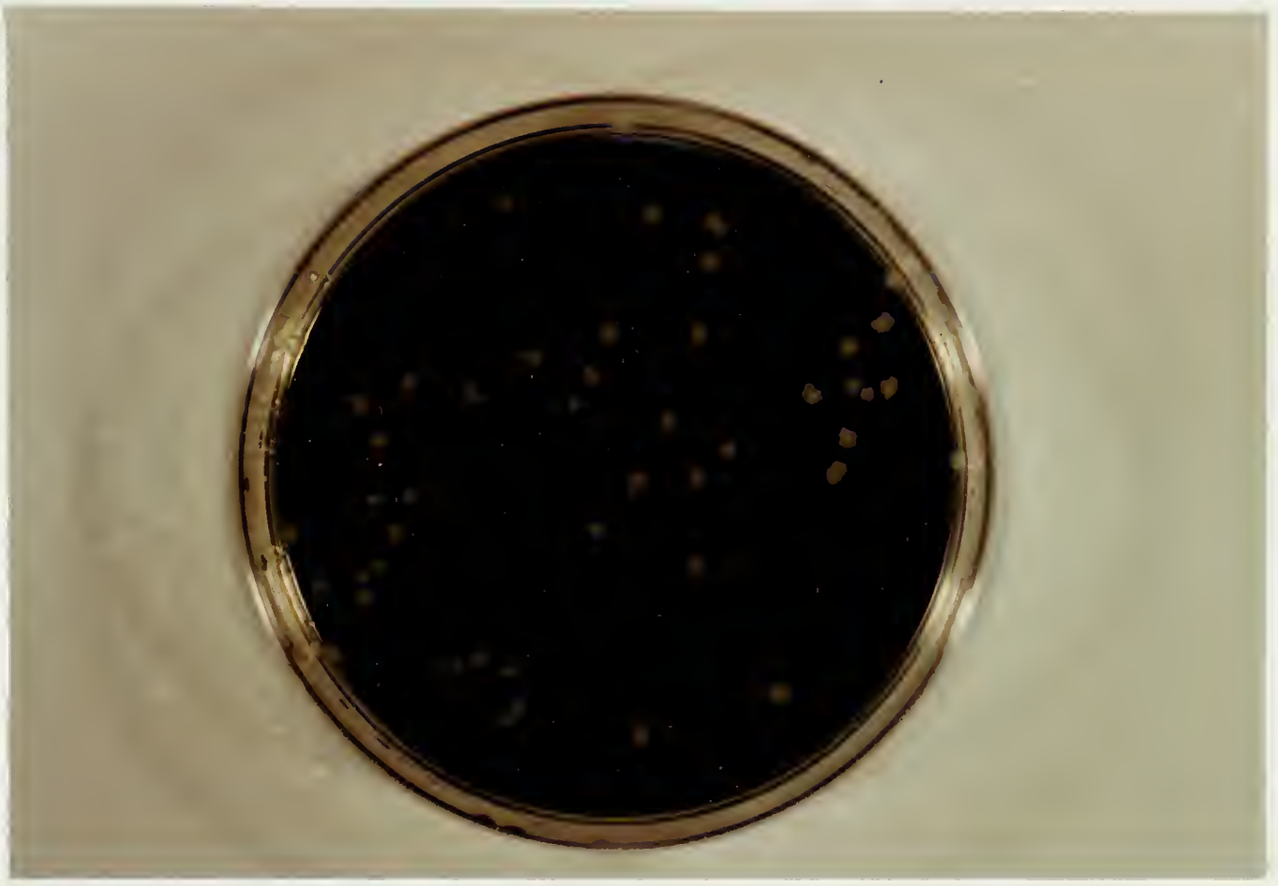
Violet red bile agar (VRBA) was used to distinguish between lactose and non-lactose fermenting colonies. Lactose fermenting colonies were a purplish red colour, generally surrounded by a ring of bile precipitate, while non-lactose fermenting colonies were colourless or cream-coloured (Difco, 1974; Ng and Stiles, 1978). VRBA colonies with a diameter of less than 0.5mm rarely form bile precipitate on VRBA or produce gas from lactose (Jones, Gibson, and Cheng, 1966). Small VRBA colonies which did not precipitate bile were selected for further study as potential variants. The technique included the inoculation of prepoured VRBA plates, and overlayering with 5ml of VRBA (Difco, 1974).

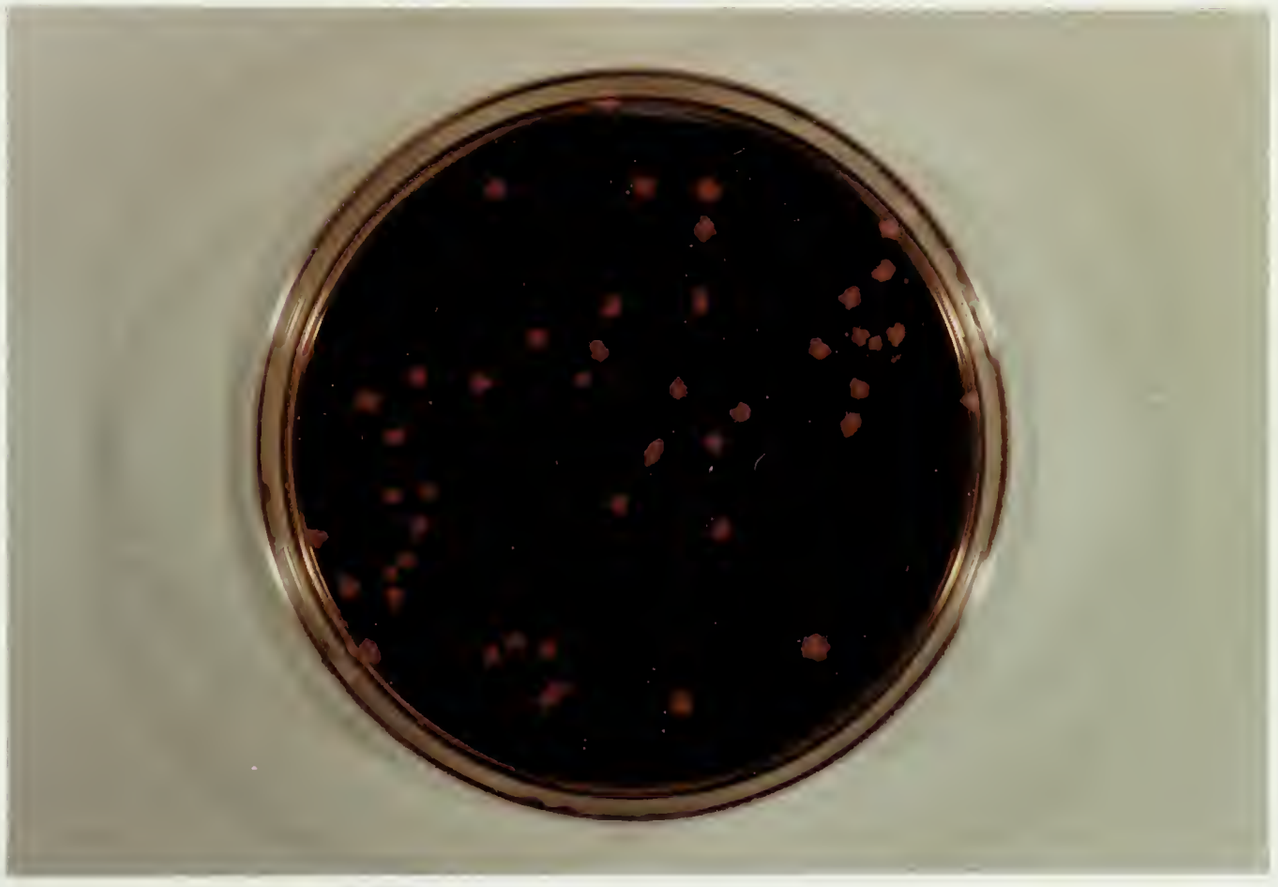
Tryptone bile agar (TBA) was used to screen cells for indole production. TBA was prepared according to the

following formula: 20.0g tryptone, 1.5g bile salts, 15.0g agar, in 1 liter of distilled water (Anderson and Baird-Parker, 1975). The modified procedure used by Stiles and Ng (1980) which involved direct inoculation of cultures onto prepoured TBA plates, rather than the conventional cellulose acetate membrane technique, was used in this study. This modification was satisfactory provided that the colonies were well-separated and there were not more than 150 colonies per plate.

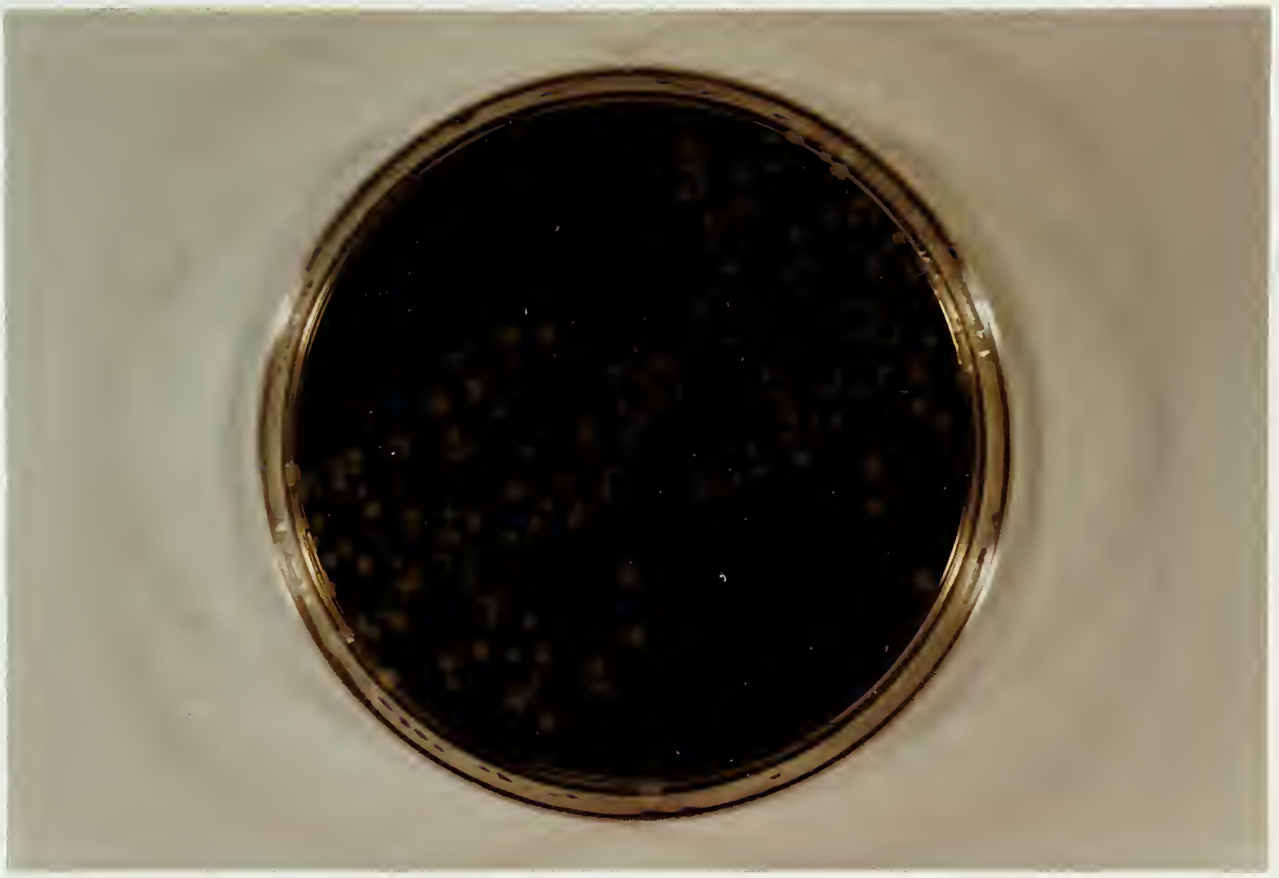
Colonies grown on TBA for 48 h were replica-plated to new TBA plates, using a sterile 12.5cm qualitative Whatman#1 filter paper. Both the original master and replica TBA plates were grown for 12 h at the original incubation temperature (35.0 or 45.0°C). The master plates were then flooded with TBA stain of the following composition, based on Anderson and Baird-Parker (1975): 2.0g *p*-dimethylamino-*benzaldehyde*, 92.0ml distilled water, and 8.0ml concentrated HCl. After 1 minute the excess stain was drained off and the stained TBA master plates were viewed against a black background. Indole-positive colonies stained a reddish-pink, while indole-negative colonies stained yellow (Anderson and Baird-Parker, 1975; Stiles and Ng, 1980). Typical examples of this colour development are shown in photographic Plates 1 to 4. Suspected indole variants on the stained master TBA plate were selected from the corresponding replica plate and used for further analysis.

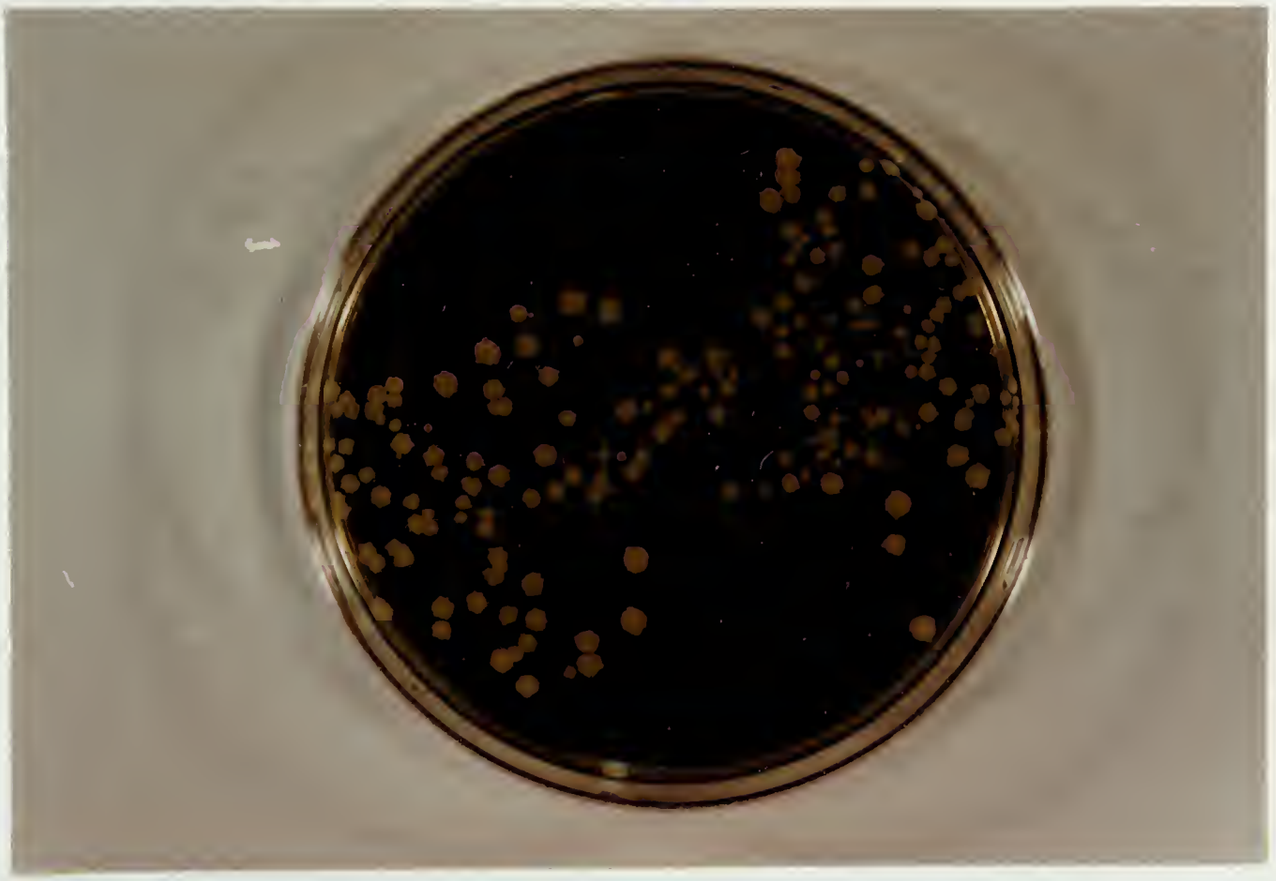
Plates 1 and 2. *E. coli* ATCC 11775 (Ind^+) culture on TBA grown at 35.0°C for 48 h, viewed against a black background, before staining (above) and after staining (below)





Plates 3 and 4. *K. pneumoniae* ATCC 13883 (Ind^-) culture on TBA grown at 35.0°C for 48 h, viewed against a black background, before staining (above) and after staining (below)





E. Detection of Culture Variants

For the heat, UV, extended storage, cold storage, and nutritional study treatments, a common screening technique was used to detect variants. Cultures were serially diluted in 9ml of sterile 0.1% (w/v) peptone water blanks, and surface streaked onto two sets of NA, TBA and VRBA plates in triplicate. One set of plates was incubated at 35.0°C, and the other at 45.0°C, for 48 h.

After incubation, the number of colonies isolated on each medium at each temperature was recorded, and their reactions on TBA and VRBA were noted. Any unusual colonies were selected from these plates, as well as a minimum of 3 random or smaller colonies from NA plates for each culture with each treatment. In the case of treatments that gave a high incidence of variants, a composite inoculum of 10 to 20 colonies was also tested. The composite inoculum was inoculated into tubes of TSI, TSB, EC, and lactose broth, and incubated at 35.0 and 45.0°C for 48 h. Any variants were held for further study by storing them on NA slants at 4.0°C.

Colonies from NA, TBA, and VRBA plates selected for further study were inoculated into tubes containing TSB, TSI, EC, and lactose broth, and incubated at 35.0 and 45.0°C for 48 h. The TSB cultures were stained with Kovac's reagent, to check for indole production. TSI cultures were checked for the ability to produce H_2S and to ferment glucose, lactose, and sucrose. EC and lactose broth cultures

were used to check for gas production from lactose, measured by gas accumulation in Durham tubes. Any variants confirmed by these tube tests were inoculated onto NA, TBA, and VRBA plates, to determine morphological changes on these media. Variants were inoculated onto NA slants, incubated for 24 h at 35.0°C, and stored at 4.0°C.

F. Comparative Analyses of *E. coli* Stock Cultures And Variants

A representative cross-section of these variants, listed in the Results section (p. 62), was selected for comparative analyses with the *E. coli* stock cultures.

Biochemical Reactions and Stability

Variants of *E. coli* ATCC 11775 and #1840 were purified by successive subculturing on NA plates and slants, and tested on TSI, TSB, EC, and lactose broth. This was done every 72 h for an average of two months for each variant. Initial testing was done at 45.0°C. The incubation temperature was later reduced to 44.5°C, because the critical temperature range for most variants was 44.0 to 44.5°C. Stock *E. coli* ATCC 11775 and #1840 cultures were used as positive controls. Stock and variant cultures of *E. coli* were tested using the BBL Minitek enteric system (BBL Minitek, 1979). Three separate colonies from NA plates were tested for each culture.

Antibiotic Sensitivity Testing

The Kirby-Bauer antibiotic sensitivity testing system (Bailey and Scott, 1974; Barry, 1977) was used to obtain the antibiogram for each culture. *E. coli* ATCC 25992 was used as the reference culture. Stock and variant cultures were inoculated into triplicate tubes of TSB, incubated at 35.0°C for 24 h, then serially diluted to 10^{-6} in 9ml 0.1% (w/v) peptone water blanks at 0°C. These dilutions were plated onto NA, and incubated at 35.0°C for 24 h. Colonies from these NA plates were treated according to the standard Kirby-Bauer antibiotic sensitivity testing technique (Barry, 1977), and used to swab the surface of prepared Mueller-Hinton agar plates (25ml per plate) (Difco).

The following antibiotic discs were applied to these prepared plates: ampicillin (10ug); carbenicillin (100ug); cephalothin (30ug); chloramphenicol (30ug); clindamycin (2ug); erythromycin (15ug); gentamycin (10ug); kanamycin (30ug); oxacillin (1ug); penicillin (10 units); tetracycline (30ug); and tobramycin (10ug). A total of 4 discs was applied per plate, and pressed down to ensure full surface contact. All cultures were tested in triplicate. The plates were incubated at 35.0°C for 24 h (Barry, 1977). Zones of inhibition were measured after 24 h incubation at 35.0°C. Zones of inhibition for *E. coli* ATCC 25992 were compared to those recorded in the literature, to confirm the correct functioning of this system (Bailey and Scott, 1977).

Serology

E. coli ATCC 11775 has a known serological type of $O_1K_1(L_1)H_7$, as cited in the catalogue of the National Collection of Type Cultures (PHSB, 1972). The serological type of the *E. coli* isolate #1840 was unknown. NA slants of the *E. coli* #1840 stock culture and *E. coli* var 1, 3, and 4 were sent to the Laboratory Centre for Disease Control in Ottawa, Ontario for serotyping.

Relative Growth Rate Analysis

The stock and variant cultures were inoculated into tubes containing 9ml TSB, and incubated in stationary culture at 35.0°C for 24 h. A 0.2ml inoculum of TSB culture was added to triplicate sets of spectrophotometer tubes containing 6.0ml TSB. Uninoculated TSB tubes were used as negative controls. Separate sets of tubes were incubated at 22.0, 35.0, and 44.5°C. OD_{600} values were recorded every 30 minutes. These readings were plotted against time for each culture, to determine their relative growth rates in TSB.

Temperature Limits for Growth, Indole Production, and Gas Production from Lactose

The stock and variant cultures were inoculated into TSB, EC, and lactose broth. Triplicate sets of each were incubated at temperatures ranging from 40.0 to 50.0°C at 0.5°C intervals, in HETO waterbaths with a temperature accuracy of ±0.05°C. This test was repeated 5 times, concentrating on the temperatures at which cultures lost their ability to grow, to produce indole in TSB (detected by

Kovac's reagent), or to produce gas in lactose or EC broth (detected by gas formation in Durham tubes).

G. Study of Indole Negative Variants

Confirmation of Loss of Ability to Produce Indole

The stock and variant cultures used in the previous section were inoculated into three sets of triplicate TSB tubes and onto TBA plates. One set of each was incubated at 35.0, 44.0, and 44.5°C, for 48 h, and checked for indole production. The cultures were also inoculated into tubes containing 3.0g tryptone, 3.0g tryptone + 2.5g dextrose, 3.0g tryptophan, and 3.0g tryptophan + 2.5g dextrose, to see if they could grow on a less nutritive broth than TSB. Three sets of triplicate tubes were incubated as described for the TSB cultures above. These cultures were checked for growth and indole production after 48 h incubation.

Pyruvate Assay

Pyruvate is a major by-product of the direct breakdown of tryptophan to indole (MacFaddin, 1977). Therefore, pyruvate accumulation can be used to determine the mechanism for inhibition of indole production by the variants at 44.5°C. The pyruvate assay was based on that used by Kersters and DeLey (1971). Colonies of stock and variant cultures from NA were inoculated into tubes of 9ml TSB. A triplicate set of each culture was incubated at 35.0 and 44.5°C. After 48 h incubation, each sample was centrifuged at 16,000xg at 0°C for 25 minutes, to obtain the culture

supernatant (Kersters and DeLey, 1971).

A 10ul aliquot of each culture supernatant was applied to a Whatman chromatography paper 3MM strip. This chromatogram was developed with a *n*-propanol - ammonia (specific gravity 0.88) - water (in a 6-3-1 v/v ratio) solvent for 1 h, and sprayed with a solution of 0.1% (w/v) *o*-phenylenediamine in 10% (w/v) trichloroacetic acid. The chromatogram was heated for 3 minutes at 105°C, then examined under a UV light of 360nm. Pyruvate spots give a greenish-yellow fluorescence and have an Rf value of 0.64. The pyruvate concentration of each sample was determined by comparison of spot size to a range of pyruvate standards (0.01 to 1.0 mmoles of pyruvate/ml) run in the same manner (Kersters and DeLey, 1971).

Tryptophanase Assay

Tryptophanase activity and synthesis were determined using the tryptophanase assay of Newton and Snell (1962), as modified by the Department of Microbiology, University of Alberta (Microbiology 444 Laboratory Manual, 1980). The stock and variant cultures were inoculated into tubes containing 9ml of nutrient broth. A triplicate set of each was incubated at 35.0, and 44.5°C for 24 h. A 0.2ml aliquot of the nutrient broth cultures was added to 10 x 75mm tubes containing 0.2ml of the assay mixture, consisting of 0.2M sodium phosphate buffer at pH 7.8, 0.025M tryptophan, and distilled water (1:1:6 v/v). Uninoculated nutrient broth treated in the same manner as the cultures was used as a

control. The tubes were covered with parafilm, and two sets of triplicate tubes of assay mixtures were prepared for incubation, one set was incubated at 35.0 and the other at 44.5°C. One set of each was sampled after 24 and 48 h incubation. The nutrient broth cultures were serially diluted in 9ml 0.1% (w/v) peptone water blanks at 0°C, and plated in duplicate on NA to determine culture purity.

After incubation, 0.12ml of a 25% trichloroacetic acid solution was added to each tube, and mixed well on a vortex mixer. Indole was extracted by adding 0.75ml of toluene to each tube and mixed thoroughly on a vortex mixer for 30 seconds. A 0.25ml aliquot of clear toluene layer was removed, and placed into a spectrophotometer tube, and 0.5ml of Erlich's reagent and 4.25ml of an acid alcohol reagent were added to each tube. The tubes were mixed well, and held at room temperature for 15 minutes to permit colour development. The A_{540} values were read in a Spectronic 21.

Tryptophan Pyrrolase Assay

Tryptophan pyrrolase is an enzyme that degrades tryptophan to indole (Lamanna and Mallette, 1965). The tryptophan pyrrolase assay, based on kynurenine production, which was used by Knox, Piras, and Tokuyamo (1966), and Blake and Kun (1971) was modified for use in this study, by excluding hematin and ascorbic acid from the assay mixture, and substituting cell extract for liver extract, because this was not a mammalian system. Stock and variant cultures were inoculated onto NA plates, and incubated at 35.0°C for

24 h. Random colonies (one colony per culture) were picked to inoculate three sets of six tubes containing 9ml TSB. One set of each was incubated at 35.0, 44.0, and 44.5°C for 48 h. Each culture was placed in an ice bath, centrifuged at 12,800xg at 0°C for 20 minutes in the 9RA rotor of a Sorval centrifuge. The supernatant was analyzed for tryptophan pyrrolase (Blake and Kun, 1971; Knox *et al.*, 1966).

The enzyme reaction mixture contained: 1.0ml 0.2M phosphate buffer at pH 7.0, 0.3ml 0.03M *l*-tryptophan, 2.0ml cell culture supernatant, and 0.7ml distilled water (Blake and Kun, 1971; Knox *et al.*, 1966). The blanks were prepared with an equivalent amount of uninoculated TSB. The enzyme reaction mixtures were incubated for 10 h at 37.0°C. After incubation, the tubes were placed on ice, and 2.0ml of 5% trichloroacetic acid chilled to 0°C was added to each tube (Blake and Kun, 1971; Knox *et al.*, 1966). Two tubes for each culture were combined to give adequate volume to measure their A_{360} values in a Gilford spectrophotometer, and hence to detect kynurenine (Blake and Kun, 1971; Knox *et al.*, 1966).

H. Study of Lactose(Gas) Negative Variants

Confirmation of Loss of Gas Production from Lactose

The stock and variant cultures were inoculated into three sets of triplicate tubes of lactose and EC broth. One set of each was incubated at 35.0, 44.0, and 44.5°C for 48 h, and checked for gas production. A more sensitive

determination of gas production in lactose broth at 44.5°C was done by gas chromatography. The cultures were inoculated into lactose broth in Hungate tubes (Canlab). Triplicate tubes were incubated at 35.0, 44.0, and 44.5°C for 48 h. A range of gas standards containing H_2 and CO_2 was prepared in Hungate tubes containing 6ml of lactose broth. The standards were held at room temperature for 24 h, to permit the gas to be partially absorbed by the medium.

Gas accumulation in Durham tubes was recorded, and both cultures and standards were placed in an ice bath, and 4.0ml of a 25% NaCl (w/v) in 1.0 N HCl solution was added to each tube. This reduced the pH to less than 1.0, and gave a final NaCl concentration of approximately 10%. This displaced most of the dissolved gas from the liquid medium. The tubes were shaken to remove the gas from the Durham tubes, so that most of the gas was in the head space of the Hungate tubes.

The gas chromatograph used in this study was a Model 700 Varian-Aerograph with a thermoconductivity detector. The column consisted of a 10ft x 3/8in Pourapak R 60-80 mesh in a flexible Pentub 1. The column was run with the following parameters set: carrier gas: N_2; filament current: 140 mamp; chart speed: 0.2 in/min.; temperature: 30°C. Conditions for recording the peak heights of the two gases were different, because of their different mobilities. Since the H_2 peaks occurred before the CO_2 peaks the column was switched from Attenuation 32, positive polarity to Attenuation 1, negative polarity in the middle of each run.

A 0.15 cc sample of gas from the head space of each culture or standard was injected into the column. Three tubes were tested for each culture and standard. Peak heights for H_2 and CO_2 were measured. The O_2 peaks for replicate runs were used as an internal standard, to ensure that constant aliquot sizes were injected onto the column. The concentration of H_2 or CO_2 in the headspace of each culture tube was determined from the respective standard curve, and the values were averaged for each culture at each incubation temperature.

ONPG Test

The ONPG test was used to check whether the cultures were able to transport lactose into the cell and break it down into glucose and galactose (MacFaddin, 1977). The stock and variant cultures were inoculated into tubes containing 9ml nutrient broth. Two sets of triplicate tubes were incubated at 35.0 and 44.5°C for 48 h. A 0.1ml sample of each culture was placed into small glass tubes containing 0.3ml of 0.85% saline and an ONPG disk (MacFaddin, 1977). The tubes were incubated at their original incubation temperatures for 4 h, then checked for colour development.

Voges-Proskauer Test

The cultures were tested for their ability to produce 2,3-butanediol as assayed by the Voges-Proskauer (VP) test (MacFaddin, 1977). *E. coli* normally produce acids but not 2,3-butanediol when fermenting sugars, so a positive VP test would indicate a basic change in their ability to break down

pyruvate to other acids (MacFaddin, 1977). The stock and variant cultures and a positive control culture, *K. pneumoniae* ATCC 13883, were used to inoculate tubes of MR-VP medium (Difco). Six replicate tubes of each culture were incubated at 35.0, 44.0, and 44.5°C. Half the cultures were sampled after 48 h incubation, and the remainder after 10 days.

pH Analysis

Neutral or alkaline pH can inhibit the production of gas from lactose by *E. coli* (Bovarnick, 1965). The pH of each lactose broth culture was determined, to ensure that lack of gas production was not attributable to pH. The stock and variant cultures were used to inoculate tubes of lactose and EC broth. One set of each was incubated at 35.0, 44.0, and 44.5°C for 48 h. Gas production and pH were recorded for each culture, and for a corresponding set of uninoculated controls. This test was repeated 3 times, and the results averaged.

At the same time, the *E. coli* cultures were inoculated into lactose tubes adjusted to pH 4.8 with an acetic acid - sodium acetate buffer at pH 4.8 (3 ml acetic acid, 4.1 g anhydrous sodium acetate, in 1 L distilled water). This test could only be done in lactose broth because the bile salts in EC broth were precipitated by the buffer. One set of triplicate tubes of each culture was incubated at 35.0, 44.0, and 44.5°C for 48 h. Gas production and pH were recorded for all tubes.

The cultures were also inoculated into unmodified lactose broth. One set of four tubes was incubated at each of 35.0, 44.0, and 44.5°C. After 24 h incubation, the amount of acetic acid required to reduce the pH of one tube of each set to pH 4.8 was determined. This amount of acetic acid was added to the other three tubes in the set. The cultures were then incubated for an additional 48 h at their respective incubation temperatures. Gas production and pH were recorded for all cultures and the uninoculated controls.

Formic Acid Assay

The accumulation of formic acid by each culture was assayed to determine if the reduced gas production by the *E. coli* variants at 44.5°C could be attributed to either a reduced production of formic acid (Kanai *et al.*, 1975), or a reduced conversion of formic acid to gas (Dawes *et al.*, 1971). The thiobarbituric acid assay for formic acid was used (Dawes, McGill, and Midgley, 1971).

The stock and variant cultures were inoculated into 9ml lactose broth in screwcapped tubes, and incubated at 35.0, 44.0, and 44.5°C for 48 h. After incubation the cultures were placed in an ice bath, and a 0.4ml aliquot of each tube was pipetted into a clean, stoppered 125 x 16 mm test tube. A 0.05ml aliquot of a 10% (w/v) ethylene glycol in water solution was added to each tube, and the tubes were held at room temperature. After 5 minutes, 0.1ml of a 5% (w/v) sodium borohydride solution in a sodium tetraborate (0.05M) - hydrochloride acid buffer at pH 8.0 was added. After an

additional 5 minutes, 0.05ml of 8.0 N sulphuric acid and 1.0ml of a 2-thiobarbituric acid solution (2.5g of 2-thiobarbiturate in 80ml of distilled water, adjusted to pH 5.4 with 2.0 N sodium hydroxide, and diluted to 100ml with distilled water) were added to each tube. The tubes were placed in a 100°C waterbath for 20 minutes for colour development (Dawes *et al.*, 1971).

The solutions were allowed to cool to room temperature. The chromophore was extracted into 1.5ml of a 5% (w/v) 11.6 N HCl in *n*-butanol solution, and centrifuged at 1000 rpm for 3 minutes to clarify the solution. The chromophore extract of two duplicate tubes was pipetted into a spectrophotometer tube. The A_{450} was determined in a Spectronic 21. Similarly treated, uninoculated lactose broth tubes were used as controls. Values obtained for similarly treated standard solutions containing 0 to 300 ug of formic acid per ml of distilled water were used to establish a standard curve for formic acid (Dawes *et al.*, 1971).

Formic Hydrogen Lyase Assay

Formic hydrogen lyase is the enzyme responsible for converting formic acid to gas in *E. coli* cultures (Dawes *et al.*, 1971). The stock and variant cultures were used to inoculate a set of triplicate tubes containing 9ml of a solution of 1% glucose, and 1% yeast extract. These tubes were incubated at 35.0°C for 12 h, to allow the cells to produce sufficient quantities of formic hydrogen lyase for assaying (Bovarnick, 1965).

The cells were harvested by centrifugation at 16,000xg at 0°C for 25 minutes, and transferred to a mortar and pestle in the presence of Alumina, Neutral A-950 (Fischer Scientific Company) and ground for 30 seconds using maximum hand pressure (Bovarnick, 1965). Formic hydrogen lyase was extracted by adding 1.5ml of deionized water per gram of "wet" cells. The mixture was centrifuged at 0°C at 16,000xg for 25 minutes. The supernatant was decanted and centrifuged for 1 h at 20,000xg at 0°C. The final supernatant contained the enzyme, and was carefully decanted into glass vials for immediate use (Bovarnick, 1965).

A 0.1ml aliquot of each enzyme extract was added to 9ml of sterile 0.1 M formate solution, containing Durham tubes, adjusted to pH 6.2 with a 0.05M sodium and potassium phosphate buffer (Bovarnick, 1965). A triplicate set of each series of tubes was incubated in waterbaths at 43.0, 44.0, 45.0, 46.0, and 47.0°C for 48 h, and gas production was recorded.

Acridine Orange Treatment

Nutrient broth cultures of stock *E. coli* ATCC 11775 and #1840 grown at 35.0°C for 12 h, were diluted with nutrient broth to obtain approximately 10^4 cells per ml, and confirmed by plating appropriate dilutions onto NA. Specific amounts of acridine orange were added to the nutrient broth cultures containing 10^4 cells, to give 0, 10, 20, 30, 40, and 50 ug acridine orange per ml. The tubes were incubated at 35.0°C for 12 h to permit plasmid curing. After

incubation, each culture was serially diluted in 0.1% (w/v) peptone water blanks at 0°C, plated onto duplicate sets of of NA, TBA, and VRBA plates in triplicate, and incubated at 35.0 and 44.5°C for 48 h. The plates were counted and examined for any unusual colonies growing on VRBA or TBA, and 20 random colonies from NA plates were selected per treatment per culture. These colonies were used to inoculate tubes of TSI, TSB, EC and lactose broth. The tubes were incubated at 35.0 and 44.5°C for 48 h to detect variants.

Confirmed lactose variants obtained by acridine orange treatment were subcultured in tubes of TSI, TSB, EC and lactose broth, and incubated at 35.0 and 44.5°C for 48 h. The subculturing was repeated every 48 h, until the ability to ferment lactose was recovered. Variants that recovered the ability to ferment lactose with the production of acid and gas at 35.0 and 44.5°C were re-treated with acridine orange, using the same technique as specified above, with dye concentrations of 0, 20, 40, 60, 80, and 100 ug acridine orange per ml. Lactose variants obtained from the re-treated, recovered lactose variants were subcultured in tubes of TSI, TSB, EC, and lactose broth, and incubated at 35.0 and 44.5°C for 48 h. Subculturing was repeated every 48 h, until the new variants recovered their original ability to ferment lactose.

IV. Results

A. Incidence of Variants with Various Treatments

The Minitek code number obtained for each stock culture (based on their biochemical reactions at 35.0°C), and the most probable identification based on that code is shown in Table 2. The most probable identity is given as a percentage certainty of identity obtained from the BBL Minitek Numerical Identification System for the Enterobacteriaceae (BBL Minitek, 1979). All monthly tests produced the same results as those listed in Table 2. In addition, standard tube tests for H_2S and indole production, gas production in EC broth, and fermentation of glucose, lactose, and sucrose by the stock cultures when grown in TSI, TSB, EC, and lactose broth at 35.0 and 44.5°C for 48 h, were conducted. These results are recorded in Table 3. All weekly tests throughout the experiment produced the same results as those listed in Table 3. Both the Minitek and standard tube tests indicated that all of the stock cultures remained consistent for these biochemical reactions throughout the 300 day duration of this experiment.

No variants were detected for any of the stock cultures stored on NA at 4.0°C. The number of colonies recovered on TBA was consistently 1/2 to 2/3 of that on NA. The number of colonies recovered on VRBA was usually 1/4 of that on NA. This observation remained constant for the stock cultures plated for all treatments throughout the study. The

TABLE 2. Identification of stock cultures on the basis of Minitek biochemical reactions at 35.0°C.

(tests done monthly; all results identical to those below)

Culture	Minitek Number	Certainty Of Identification[1]
C. freundii	5041157	99.93%
E. cloacae	4367777	99.98%
E. aerogenes	4353377	96.58%
E. hafniae	4353503	99.87%
E. coli	4453316	99.90%
K. pneumoniae	4371737	99.89%
S. choleraesuis	4003012	98.44%
S. typhimurium	5011010	99.97%
E. coli #1840	4453316	99.90%
E. agglomerans	4361527	96.58%

[1]Percentage certainty of identification, based on the BBL Minitek Enteric System (BBL Minitek, 1979).

TABLE 3. Summary of the tested biochemical characteristics of stock cultures incubated 48 h at 35.0 and 44.5°C.

(tests done weekly; all results same as shown below)

Culture	H_2S	Ind	EC	Glu	Lac	Suc
ATCC Cultures						
C. freundii						
35.0°C	+	-	g	a,g	a,g	a,g
44.5°C	+	-	-	a,g	a	a
E. cloacae						
35.0°C	-	-	g	a,g	a,g	a,g
44.5°C	-	-	-	a,g	a	a
E. aerogenes						
35.0°C	-	-	g	a,g	a,g	a,g
44.5°C	-	-	-	a,g	a	a
E. hafniae						
35.0°C	-	-	-	a,g	-	-
44.5°C	-	-	-	a	-	-
E. coli						
35.0°C	-	+	g	a,g	a,g	a,g
44.5°C	-	+	g	a,g	a,g	a,g
K. pneumoniae						
35.0°C	-	-	g	a,g	a,g	a,g
44.5°C	-	-	-	a,g	a	a
S. choleraesuis						
35.0°C	-	-	-	a,g	-	-
44.5°C	-	-	-	a	-	-
S. typhimurium						
35.0°C	+	-	-	a,g	-	-
44.5°C	+	-	-	a	-	-
Meat Isolates						
E. coli #1840						
35.0°C	-	+	g	a,g	a,g	a,g
44.5°C	-	+	g	a,g	a,g	a,g
E. agglomerans						
35.0°C	-	-	g	a,g	a,g	a,g
44.5°C	-	-	-	a,g	a	a

prolonged storage of *E. coli* ATCC 11775 and #1840 stored on NA slants at 4.0°C for 166 days did not result in any changes in culture characteristics. They remained $Ind^+EC^+Glu^+Lac^+Suc(a,g)^+$ at 35.0 and 45.0°C throughout the study. The results for the production of variants due to the specific treatments are summarized in Table 4. More detailed information of the results is given for each treatment below.

Heat Treatment

Heating at 52.0°C for 15 minutes produced a 1,000-fold decrease in the number of colonies per ml recovered on NA and TBA, and a 10,000-fold decrease in the number of colonies per ml recovered on VRBA. However, all isolates from the heat-treated and unheated control cultures exhibited the same biochemical characteristics for H_2S, indole, EC, glucose, lactose, and sucrose at 35.0 and 45.0°C. The H_2S^+ reaction of *C. freundii* ATCC 8090 at 44.5°C weakened over the course of this experiment, but it was not lost. No variants were detected throughout the 124 days of this study.

Extended Storage Treatment

The *E. coli* ATCC 11775 and #1840 TSB broth cultures died out after 52 days of storage at 45.0°C, dried out after 75 days storage at 35.0°C, and after 101 days storage at 4.0°C, all of the culture had been used in the tests. For the cultures stored at 45.0°C, after 6 days of storage, an *E. coli* ATCC 11775 colony which did not precipitate bile on

TABLE 4. Summary of the 44.5°C variants and the treatments from which they were obtained.

Treatment	*E. coli* Variants Found	Time Required (days)	Type of 44.5°C Variant
Extended Storage			
45.0°C	ATCC	6	$Ind^-EC^-Lac(g)^-$
35.0°C	ATCC	75	$Ind^+EC^-Lac(g)^-$
35.0°C	#1840	68,75	$Ind^+EC^-Lac(g)^-$
4.0°C	ATCC	75,76	$Ind^+EC^-Lac(g)^-$
4.0°C	#1840	68,75,76	$Ind^+EC^-Lac(g)^-$
Frozen Storage			
-16°C	ATCC	59	$Ind^-EC^-Lac(g)^-$
	ATCC	70	$Ind^+EC^-Lac(g)^-$
Sloppy Agar			
4.0°C and -16°C	ATCC and #1840	5*	$Ind^+EC^-Lac(g)^-$
Nutritional Semisolids			
4.0°C[1]	ATCC and #1840	11*	$Ind^+EC^-Lac(g)^-$
Nutritional Broths			
-16°C	ATCC and #1840	11*	$Ind^+EC^-Lac(g)^-$
UV	ATCC	45	$Ind^-EC^-Lac(g)^-$

*First found at time noted, then each time thereafter
[1]All media except TSB 30g/L produced variants
[2]All media except those with greater than TSB 3g/L, which did not exclude soytone, produced variants

VRBA at 45.0°C, was detected. After 3 subcultures, this variant recovered its ability to precipitate bile at 45.0°C, but remained smaller in size on VRBA compared to the normal colonies. Initially this variant was unable to produce either gas from lactose or indole from tryptophan at 45.0°C. The exact temperature at which these functions were lost was 44.5°C. All subsequent subcultures were $Ind^+EC^+Lac(a,g)^+$ at 44.0°C, and $Ind^-EC^-Lac(g)^-$ at 44.5°C. No other variants were detected for the cultures stored at 45.0°C, throughout the 52 days of storage at 45.0°C.

The variants obtained for the *E. coli* cultures stored at 35.0°C are shown in Table 4. These variants were detected among the randomly selected isolates from NA. The variants were $Ind^+EC^+Lac(a,g)^+$ at 44.0°C, and $Ind^+EC^-Lac(g)^-$ at 44.5°C. No other variants were detected from the cultures stored at 35.0°C, throughout the 75 days of storage. The variants obtained for the *E. coli* cultures stored at 4.0°C are also shown in Table 4. These variants were randomly selected isolates from NA. They were $Ind^+EC^+Lac(a,g)^+$ at 44.0°C, and $Ind^+EC^-Lac(g)^-$ at 44.5°C. No other variants were detected for any cultures stored at 4.0°C, throughout the 101 days of storage at 4.0°C.

Frozen Storage Treatment

Overnight frozen storage in TSB at -16°C generally produced a 100-fold decrease in the number of cells per ml recovered on NA, TBA, and VRBA. With continued frozen storage, this decrease became 4 log cycles or more. The H_2S^+

reactions of *C. freundii* and *S. typhimurium* at 44.5°C, measured on TSI slants, weakened with continued frozen storage, but this characteristic was not lost.

After 59 days of frozen storage, an *E. coli* ATCC 11775 variant was detected among the randomly selected isolates from NA. Initially, this variant failed to produce either gas from lactose or EC broth, or indole from tryptophan at 44.5°C. All subcultures thereafter were $Ind^{+}EC^{+}Lac(a,g)^{+}$ at 44.0°C, and $Ind^{-}EC^{-}Lac(g)^{-}$ at 44.5°C. After 70 days of cold storage, another *E. coli* ATCC 11775 variant was detected. This variant was also among the randomly selected colonies from NA. Initially this variant failed to produce gas from lactose or EC broth (Durham tube method) at 44.5°C. All subcultures thereafter remained $Ind^{+}EC^{+}Lac(a,g)^{+}$ at 44.0°C, and $Ind^{+}EC^{-}Lac(g)^{-}$ at 44.5°C. No other variants were detected throughout the 204 days of this treatment.

Sloppy Agar Cultures

Overnight storage of sloppy agar cultures at 4.0 and -16°C produced a 2 log cycle decrease in the number of colonies per ml recovered on NA, TBA, and VRBA. This became a 3 log cycle decrease over the 142 day course of this experiment. Variants of *E. coli* ATCC 11775 and #1840 grown on sloppy agar were first detected after 5 days of storage at 4.0 and -16°C. Similar variants were detected each week thereafter for this treatment. In later samples, as many as 20 colonies were inoculated into a single series of tubes and gave the same reactions. It appeared, therefore, that

most or all of the cells in these *E. coli* cultures had become variants. All the variants were randomly selected isolates from NA. All subcultures tested were $Ind^{+}EC^{+}Lac(a,g)^{+}$ at 44.0°C, and $Ind^{+}EC^{-}Lac(g)^{-}$ at 44.5°C. No other variants were found for any of the other cultures tested throughout the 142 days of this experiment. The production of H_2S by *C. freundii* on TSI slants weakened after storage at 4.0 and -16°C, but the characteristic was not lost.

Nutritional Study

Overnight storage of the *E. coli* stock cultures at 4.0 and -16°C resulted in a 100-fold decrease in the number of colonies per ml recovered on NA, TBA, and VRBA. This increased to a 3 log cycle decrease by the end of this study. For the cultures stored at 4.0°C in semisolid agars (see medium composition in Table 1) variants of *E. coli* ATCC 11775 and #1840 were detected on all media with the exception of agar containing 30g TSB/L. Variants were first isolated after 11 days of storage at 4.0°C, and each week thereafter. Later samples gave similar results when up to 20 colonies were used to inoculate a single series of tubes. All the variants were randomly selected isolates from NA. All subcultures examined were $Ind^{+}EC^{+}Lac(a,g)^{+}$ at 44.0°C, and $Ind^{+}EC^{-}Lac(g)^{-}$ at 44.5°C.

For the cultures stored at -16°C in broths (see medium composition in Table 1) variants of *E. coli* ATCC 11775 and #1840 were detected from all broths, except those which

contained greater than 3g TSB/L, and did not exclude soytone. Variants were first detected after 11 days storage at -16°C, and each week thereafter. The characteristic variants were still detected in later samples when up to 20 colonies were used to inoculate a single series of tubes. All variants isolated were randomly selected isolates from NA. All subcultures tested were $Ind^{+}EC^{+}Lac(a,g)^{+}$ at 44.0°C, and $Ind^{+}EC^{-}Lac(g)^{-}$ at 44.5°C. No other variants were detected for any culture tested throughout the 112 days of this experiment.

UV Treatment

Daily irradiation for 5 minutes with a germicidal UV lamp caused a 10,000-fold decrease in the number of cells per ml recovered on NA, TBA, and VRBA. After 45 successive exposures to UV treatment, three *E. coli* ATCC 11775 colonies were detected which did not precipitate bile on VRBA at 45.0°C. The ability to precipitate bile on VRBA at 45.0°C was restored after 4 subcultures, but they remained smaller than normal colonies when grown on VRBA at 45.0°C.

Upon initial isolation the variants were unable to produce either gas from lactose or indole from tryptophan at 45.0°C (using both the TBA and Kovac's methods). However, the exact temperature at which the functions were lost was found to be 44.5°C. All subcultures since then remained $Ind^{+}EC^{+}Lac(a,g)^{+}$ at 44.0°C, and $Ind^{-}EC^{-}Lac(g)^{-}$ at 44.5°C. No other variants were obtained for any of the cultures treated throughout the 93 days of this study.

B. Comparative Analyses of *E. coli* Stock and Variant Cultures

A representative sample of the *E. coli* variants was selected to confirm that the variants were derived from the stock cultures. They included one Lac(g)$^-$ variant from each treatment, and all 3 Ind$^-$ variants. The number (#1) appended to the variants listed below indicates that it was the first variant isolated from that source.

E. coli ATCC 11775 variants

var 1: 4.0°C sloppy agar #1

var 2: -16°C sloppy agar #1

var 3: 4.0°C extended storage

var 4: -16°C 3g/L TSB broth #1

var 5: 4.0°C 3g/L TSB semisolid #1

var 6: 5 min. UV irradiation

var 7: 45.0°C extended storage

var 8: cold storage

E. coli isolate #1840 from meat variants

var 9: 4.0°C sloppy agar #1

var 10: -16°C sloppy agar #1

var 11: 4.0°C extended storage

var 12: -16°C 3g/L TSB broth #1

var 13: 4.0°C 3g/L TSB semisolid #1

General Biochemical Reactions at 35.0°C

Both *E. coli* ATCC 11775 and #1840 stock cultures exhibited the following biochemical characteristics at 35.0°C, when tested by the BBL Minitek method: nitrate

reductase positive; phenylalanine deaminase negative; H_2S production negative; indole production positive; Voges-Proskauer reaction negative; citrate utilization negative; ONPG negative; urease negative; lysine decarboxylase positive; arginine dihydrolase negative; ornithine decarboxylase positive; able to ferment dextrose, adonitol, arabinose, sorbitol, and rhamnose; and unable to ferment malonate, inositol, raffinose, and sucrose. The biochemical reactions were determined in triplicate at monthly intervals for both of the *E. coli* stock cultures. Both of the *E. coli* stock cultures had the same Minitek code number throughout the study (see Table 2). The biochemical tests run in triplicate at 35.0°C on the *E. coli* variants, gave the same results as the stock cultures.

Serology

E. coli ATCC 11775 has a known serotype of $O_1:K_1(L_1):H_7$ (PHSB, 1972). The three variants of *E. coli* ATCC 11775 tested (var 1, 3, and 4) had a serotype of $O_1:K_1:H_7$. The stock culture of *E. coli* #1840 was also serotyped. It had a serotype of O?:K?:H_2. Since its O and K antigens could not be typed by available antisera, no specific serotype could be established for this organism, and no further serotyping of these variants was done.

Antibiotic Sensitivity Testing

The antibiograms obtained for the *E. coli* ATCC 11775 and #1840 stock and variant cultures, by the Kirby-Bauer antibiotic sensitivity testing method, are recorded in Table

5. The antibiotic reactions for the control *E. coli* ATCC 25922 culture correspondended with those reported in the literature, indicating that the antibiotic testing system was working properly (Bailey and Scott, 1974; Barry, 1977). All variants tested gave the same antibiogram as the stock cultures from which they were derived. The antibiograms enabled the *E. coli* ATCC 11775 and #1840 cultures to be distinguished as separate cultures, as they differed in their sensitivity to erythromycin and cephalothin.

Relative Growth Rates

Growth rates of the stock cultures were compared with *E. coli* variants. The variants had approximately the same growth rates in TSB at 22.0, 35.0, and 45.0°C as the stock cultures from which they were derived. All *E. coli* #1840 cultures had lower growth rates at each incubation temperature than the *E. coli* ATCC 11775 cultures. *E. coli* ATCC 11775 stock and variant TSB cultures incubated at 22.0, 35.0, and 45.0°C had generation times of 20.4, 10.7, and 12.1 minutes, respectively. *E. coli* #1840 stock and variant TSB cultures incubated at 22.0, 35.0, and 45.0°C had generation times of 29.0, 11.2, and 12.5 minutes, respectively.

Temperature Limits for Growth, Indole Production, and Production of Gas from Lactose

The temperature limits for growth, indole production, and gas production from lactose for the *E. coli* stock and variant cultures are listed in Table 6. All *E. coli* cultures

TABLE 5. Kirby-Bauer antibiotic sensitivity results obtained for *E. coli* ATCC 11775 and isolate #1840 stock and variant cultures.

(tests done in triplicate; all results same as shown below)

	Antibiotic Sensitivity Results[1]											
Culture	1	2	3	4	5	6	7	8	9	10	11	12
E. coli ATCC 25922												
literature	*	s	s	*	r	*	s	r	s	*	s	s
experimental	s	s	s	s	r	r	s	r	s	r	s	s
E. coli ATCC 11775												
stock	s	s	s	s	r	r	s	r	s	r	s	s
var 1	s	s	s	s	r	r	s	r	s	r	s	s
var 2	s	s	s	s	r	r	s	r	s	r	s	s
var 3	s	s	s	s	r	r	s	r	s	r	s	s
var 4	s	s	s	s	r	r	s	r	s	r	s	s
var 5	s	s	s	s	r	r	s	r	s	r	s	s
var 6	s	s	s	s	r	r	s	r	s	r	s	s
var 7	s	s	s	s	r	r	s	r	s	r	s	s
var 8	s	s	s	s	r	r	s	r	s	r	s	s
E. coli #1840												
stock	s	s	s	s	s	r	s	r	i	r	s	s
var 9	s	s	s	s	s	r	s	r	i	r	s	s
var 10	s	s	s	s	s	r	s	r	i	r	s	s
var 11	s	s	s	s	s	r	s	r	i	r	s	s
var 12	s	s	s	s	s	r	s	r	i	r	s	s
var 13	s	s	s	s	s	r	s	r	i	r	s	s

[1]KEY

Antibiotic Discs Used

1 carbenicillin (100ug)
2 tetracycline (30ug)
3 kanamycin (30ug)
4 tobramycin (10ug)
5 erythromycin (15ug)
6 clindamycin (2ug)
7 gentamicin (10ug)
8 penicillin (10 units)
9 cephalothin (30ug)
10 oxacillin (1ug)
11 ampicillin (10ug)
12 chloramphenicol (30ug)

Antibiotic Sensitivity Reactions

* no result given in literature
r resistant to drug in given concentration
s sensitive to drug in given concentration
i intermediate between sensitive and resistant

TABLE 6. Temperatures at which *E. coli* ATCC 11775 and isolate #1840 stock and variant cultures lost their ability to grow, to produce indole, and to produce gas from lactose.

(tests done in triplicate; all results same as shown below)

	Temperature At Which Function Was Lost		
Cultures	Growth	indole	Lactose(gas)
E. coli ATCC 11775			
stock	48.5	48.0	47.0
var 1	48.5	47.5	44.5
var 2	48.5	47.5	44.5
var 3	48.5	47.5	44.5
var 4	48.5	47.5	44.5
var 5	48.5	47.5	44.5
var 6	48.5	44.5	44.5
var 7	48.5	44.5	44.5
var 8	48.5	44.5	44.5
E. coli #1840			
stock	48.5	48.0	47.0
var 9	48.5	47.5	44.5
var 10	48.5	47.5	44.5
var 11	48.5	47.5	44.5
var 12	48.5	47.5	44.5
var 13	48.5	47.5	44.5

tested lost the ability to grow in TSB, EC, and lactose broth at 48.5°C. No variations in maximum growth temperature were observed. The ability to produce indole in TSB (Kovac's method) was lost by both *E. coli* stock cultures at 48.0°C, and at 47.5°C by *E. coli* Ind^+ variants. However, the *E. coli* ATCC 11775 Ind^- var 6, 7, and 8 variants stopped producing indole at 44.5°C, markedly lower than the other *E. coli* cultures. The ability to produce gas from lactose in EC and lactose broth was lost at 47.0°C by both of the *E. coli* stock cultures. All *E. coli* Lac^- variants lost the ability to produce gas, but not acid, at 44.5°C.

C. Study of Indole Negative Variants

Confirmation of Loss of Indole Production

E. coli ATCC 11775 and #1840 stock and Ind^+ variant cultures produced indole at 35.0, 44.0, and 44.5°C (TBA and Kovac's methods). However, three *E. coli* ATCC 11775 Ind^- variants (var 6, 7, and 8) produced indole actively at 35.0°C, weakly at 44.0°C, and not at all at 44.5°C.

Tryptophan Pyrrolase Assay

The results of the tryptophan pyrrolase assay are shown in Table 7. Because of the low levels of enzyme produced, the assay time was extended to 10 h. The enzyme activity of the extracts obtained from cultures incubated at 44.5°C was approximately half that observed for the same cultures incubated at 35.0°C. Enzyme extracts from *E. coli* variants had approximately half the activity of extracts from stock

TABLE 7. Summary of the A_{360} values for tryptophan pyrrolase assays (10 h) on extracts from *E. coli* ATCC 11775 and isolate #1840 stock and variant cultures incubated 48 h at 35.0, 44.0, and 44.5°C.

(tests done in triplicate; averaged results shown below)

	A_{360} Values Obtained (reflect kynurenine accumulation due to enzyme activity)		
Culture	35.0°C	44.0°C	44.5°C
E. coli ATCC 11775			
stock	0.552	0.266	0.225
var 1	0.254	0.155	0.118
var 2	0.251	0.152	0.116
var 3	0.264	0.175	0.123
var 4	0.248	0.144	0.119
var 5	0.255	0.163	0.121
var 6	0.200	0.109	0.098
var 7	0.210	0.122	0.106
var 8	0.204	0.112	0.102
E. coli #1840			
stock	0.483	0.235	0.212
var 9	0.209	0.103	0.085
var 10	0.208	0.101	0.084
var 11	0.211	0.106	0.088
var 12	0.204	0.092	0.093
var 13	0.205	0.098	0.099
uninoculated control	0.000	0.000	0.000

standard deviation ±0.001

cultures. There was no noticable difference in tryptophan pyrrolase activity between enzyme extracts obtained from Ind^+ and Ind^- *E. coli* variants, when assayed at 37.0°C. The temperature-sensitivity of the enzyme itself could not be accurately assessed, due to the limitations of the assay which had been developed for mammalian enzymes at 37.0°C, and due to the lack of a standard tryptophan pyrrolase sample needed to check the reliability of this assay at elevated temperatures (Knox *et al.*, 1966; Blake and Kun, 1971).

Pyruvate Assay

The results for the pyruvate assays on *E. coli* ATCC 11775 cultures are shown in Table 8. At 35.0°C, all cultures were Ind^+ and had approximately the same amount of pyruvate accumulation. Less pyruvate was accumulated at 44.5°C than at 35.0°C. At 44.5°C, pyruvate accumulation was greatest for the stock cultures, slightly lower for most variants, and lowest for the 44.5°C Ind^- variants, suggesting that pyruvate accumulation decreased as indole production decreased. However, the small differences in pyruvate accumulation by the indole and non-indole producing variants at 44.5°C limited the importance that could be attached to this observation. Interference from other sources of pyruvate and utilization of pyruvate in other metabolic pathways could not be eliminated.

TABLE 8. Summary of the indole reaction of TSB cultures of *E. coli* ATCC 11775 stock and variant cultures after incubating for 48 h at 35.0 and 44.5°C, and amount of pyruvate accumulated by each culture.[1]

(tests done in triplicate; averaged results shown below)

	35.0°C, 48 h		44.5°C, 48 h	
	indole	Pyruvate	indole	Pyruvate
Culture		(mmoles/ml)		(mmoles/ml)
E. coli ATCC 11775				
stock	+	0.10	+	0.06
var 1	+	0.10	w	0.05
var 2	+	0.09	w	0.05
var 3	+	0.09	w	0.05
var 4	+	0.10	w	0.04
var 5	+	0.10	w	0.04
var 6	+	0.09	-	0.03
var 7	+	0.09	-	0.02
var 8	+	0.09	-	0.03
uninoculated TSB	-	0.00	-	0.00

Key

w reactions were weak, but still positive

standard deviation ±0.004

Tryptophanase Assay

The results of the tryptophanase assay conducted on the *E. coli* ATCC stock and variant cultures are shown in Tables 9 and 10. Cultures produced more indole when grown at 35.0°C than at 44.5°C, and more indole was detected when assayed at 35.0°C than at 44.5°C. From these data, it appeared that the synthesis of tryptophanase at 44.5°C was slightly temperature-sensitive, while the activity of tryptophanase was markedly temperature-sensitive. These temperature-sensitivities were most noticable for the 44.5°C Ind$^-$ *E. coli* variants (var 6, 7, and 8) which exhibited no enzyme activity when assayed at 44.5°C. As the A_{540} values obtained after a 48-h assay were only slightly higher than those obtained after a 24-h assay (see Tables 9 and 10), it was decided that further extension of the assay incubation would not aid in detecting indole production at 44.5°C by the *E. coli* indole variants.

D. Study of Lactose(Gas) Negative Variants

Confirmation of Loss of Gas Production from Lactose

E. coli ATCC 11775 and #1840 stock and variant cultures produced gas in EC and lactose broth media (Durham tube method) at 35.0 and 44.0°C. Less gas was produced at 44.0°C than at 35.0°C. The stock cultures produced gas in these media at 44.5°C. The Lac(g)$^-$ variants grew but did not produce gas.

TABLE 9. Summary of the data for 24-h tryptophanase assays at 35.0 and 44.5°C on *E. coli* ATCC 11775 stock and variant cultures grown at 35.0 and 44.5°C in nutrient broth.

(tests done in triplicate; averaged results shown below)

	A_{540} Readings Obtained			
	35.0° Culture		44.5°C Culture	
Culture	35.0°C	44.5°C	35.0°C	44.5°C
E. coli ATCC 11775				
stock	0.349	0.170	0.297	0.153
var 1	0.339	0.168	0.247	0.157
var 2	0.273	0.117	0.185	0.098
var 3	0.247	0.147	0.270	0.134
var 4	0.278	0.143	0.272	0.136
var 5	0.263	0.133	0.241	0.117
var 6	0.246	0.000	0.038	0.000
var 7	0.281	0.000	0.123	0.000
var 8	0.280	0.000	0.096	0.000
uninoculated broth	0.000	0.000	0.000	0.000

standard deviation ±0.002

TABLE 10. Summary of the data for 48-h tryptophanase assays at 35.0 and 44.5°C on *E. coli* ATCC 11775 stock and variant cultures grown at 35.0 and 44.5°C in nutrient broth.

(tests done in triplicate; averaged results shown below)

	A_{540} Values Obtained			
	35.0°C		44.5°C	
Culture	35.0°C	44.5°C	35.0°C	44.5°C
E. coli ATCC 11775				
stock	0.419	0.242	0.350	0.185
var 1	0.409	0.230	0.330	0.173
var 2	0.327	0.191	0.303	0.140
var 3	0.310	0.198	0.313	0.166
var 4	0.344	0.205	0.320	0.108
var 5	0.324	0.191	0.315	0.146
var 6	0.306	0.000	0.077	0.000
var 7	0.349	0.000	0.284	0.000
var 8	0.353	0.000	0.200	0.000
uninoculated broth	0.000	0.000	0.000	0.000

standard deviation ±0.002

Gas chromatography was used for a more exact analysis of gas production. The volumes of H_2 and CO_2 produced by each *E. coli* culture at 35.0, 44.0, and 44.5°C in lactose broth are shown in Table 11. Gas production decreased as the temperature of incubation was increased. At 44.5°C, the *E. coli* Lac(g)$^-$ variants still produced H_2 and CO_2 in trace amounts, approximately 1/10th to 1/30th of the amount produced at 44.0°C or by the stock cultures at 44.5°C, too little to be detected in Durham tubes.

Ratios of H_2 to CO_2 produced at 35.0, 44.0, and 44.5°C, determined by gas chromatography, are shown in Table 12. The *E. coli* ATCC 11775 stock culture retained a consistent H_2:CO_2 ratio of approximately 1:2 at all temperatures. The H_2:CO_2 ratio for the *E. coli* #1840 stock culture was 1:3 at 35.0°C, but dropped to 1:1.5 at 44.0 and 44.5°C. The H_2:CO_2 ratios for the *E. coli* variants were approximately 1:2 at 35.0 and 44.0°C, but dropped to 1:1 at 44.5°C. However, the low levels of gas detected at the inhibitory temperatures make the accuracy of the ratios questionable.

ONPG Tests

All of the *E. coli* ATCC 11775 and #1840 stock and variant cultures were all ONPG$^+$ at 35.0 and 44.5°C.

VP Tests

The positive control *K. pneumoniae* ATCC 13883 was VP$^+$ at 35.0 and 44.5°C after both 2 and 10 days of incubation. The negative control (uninoculated broth) and the *E. coli* ATCC 11775 and #1840 stock and variant cultures were VP$^-$

TABLE 11. Amounts of H_2 and CO_2 gas produced by *E. coli* ATCC 11775 and isolate #1840 stock and variant cultures incubated at 35.0, 44.0, and 44.5°C for 48 h in lactose broth.

(tests done in triplicate; averaged results shown below)

Culture	Amounts of H_2 and CO_2 (cc gas per tube)					
	35.0°C		44.0°C		44.5°C	
	H_2	CO_2	H_2	CO_2	H_2	CO_2
E. coli ATCC 11775						
stock	2.6	4.2	1.5	3.4	1.3	2.4
var 1	2.8	3.8	1.6	3.4	0.1	0.1
var 2	3.1	5.4	2.2	3.4	0.1	0.1
var 3	3.6	5.5	1.7	3.2	0.1	0.1
var 4	2.7	5.3	1.3	3.2	0.1	0.1
var 5	2.5	5.4	1.2	2.8	0.1	0.1
var 6	3.2	4.7	1.2	2.4	0.2	0.2
var 7	2.8	4.8	1.1	2.2	0.2	0.2
var 8	3.4	5.5	0.9	2.0	0.2	0.2
E. coli #1840						
stock	1.6	4.4	2.5	3.6	2.3	3.4
var 9	2.2	3.4	1.8	3.3	0.1	0.1
var 10	1.7	3.6	1.8	2.7	0.1	0.1
var 11	1.8	4.0	1.6	2.9	0.1	0.1
var 12	2.2	5.2	1.4	3.0	0.1	0.1
var 13	2.2	3.0	2.2	2.8	0.1	0.1
uninoculated broth	0.0	0.0	0.0	0.0	0.0	0.0
K. pneumoniae ATCC 13883	2.1	0.5	not tested		0.0	0.0

standard deviation ±0.1 except CO_2 at 35.0°C ±0.2

TABLE 12. Summary of the H_2:CO_2 gas ratios for *E. coli* ATCC 11775 and isolate #1840 stock and variant lactose broth cultures incubated at 35.0, 44.0, and 44.5°C for 48 h.

(ratios calculated from data recorded in Table 11)

	H_2:CO_2 Ratios Obtained		
Culture	35.0°C	44.0°C	44.5°C
E. coli ATCC 11775			
stock	1:1.7	1:2.2	1:1.9
var 1	1:1.3	1:2.2	1:1 *
var 2	1:1.7	1:1.5	1:1 *
var 3	1:1.5	1:1.9	1:1 *
var 4	1:1.6	1:2.2	1:1 *
var 5	1:2.1	1:2.2	1:1 *
var 6	1:1.4	1:2.2	1:1 *
var 7	1:1.8	1:2.0	1:1 *
var 8	1:1.6	1:2.0	1:1 *
E. coli #1840			
stock	1:2.9	1:1.5	1:1.5
var 9	1:1.6	1:1.8	1:1 *
var 10	1:2.0	1:1.6	1:1 *
var 11	1:2.2	1:1.8	1:1 *
var 12	1:2.3	1:2.1	1:1 *
var 13	1:1.3	1:1.3	1:1 *
uninoculated broth	0	0	0
K. pneumoniae ATCC 13883	1:0.3	not tested	0

*Based on low levels of gas production

under the same test conditions.

pH Analysis

The average pH values for EC and lactose broth cultures of the *E. coli* stock and variant cultures after 48 h of incubation at 35.0, 44.0, and 44.5°C are shown in Table 13. All cultures produced less acid as the temperature of incubation was increased. All cultures attained pH values below 6, the critical pH above which the conversion of formic acid to gas may be inhibited.

The variants produced more acid than the stock cultures, especially at 35.0°C. Further study of the effect of pH was conducted at pH 4.8, the final pH of the variants grown at 35.0°C. Attempts to grow the *E. coli* cultures in a lactose broth medium buffered at pH 4.8 were unsuccessful. No growth was evident even after incubation at 35.0°C for 4 days. The acetic acid-sodium acetate buffer precipitated bile salts, so EC broth could not be used. However, lactose broth cultures of *E. coli*, adjusted to pH 4.8 by the addition of acetic acid after 24 h of incubation at 35.0, 44.0, and 44.5°C, continued to grow when incubated at their respective incubation temperatures for an additional 48 h. Variants thus treated still did not produce gas from lactose (Durham tube method) at 44.5°C.

Formic Acid Assay

The results for the formic acid assay of the *E. coli* cultures are shown in Table 14. As the temperature of incubation was changed from 35.0 to 44.5°C, formic acid

TABLE 13. pH of EC and lactose broth after growth of *E. coli* ATCC 11775 and isolate #1840 stock and variant cultures for 48 h at 35.0, 44.0, and 44.5°C.

(tests repeated on three occasions; averaged results below)

	Final pH of Lactose broth			Final pH of EC broth		
Culture	35.0°C	44.0°C	44.5°C	35.0°C	44.0°C	44.5°C
E. coli ATCC 11775						
stock	5.4	5.4	5.5	5.6	5.6	5.4
var 1	4.8	5.2	5.4	5.6	5.5	5.7
var 2	5.2	5.4	5.6	5.6	5.5	5.7
var 3	4.7	5.3	5.5	5.6	5.5	5.6
var 4	4.7	5.2	5.4	5.6	5.6	5.7
var 5	4.7	5.2	5.5	5.7	5.6	5.7
var 6	4.8	5.3	5.6	5.6	5.4	5.7
var 7	4.8	5.3	5.5	5.7	5.5	5.7
var 8	4.9	5.4	5.6	5.6	5.6	5.6
E. coli #1840						
stock	5.4	5.5	5.8	5.6	5.6	5.5
var 9	4.8	5.3	5.4	5.5	5.4	5.7
var 10	4.7	5.4	5.6	5.6	5.5	5.6
var 11	4.8	5.4	5.5	5.6	5.5	5.6
var 12	4.8	5.3	5.5	5.5	5.5	5.7
var 13	4.9	5.4	5.6	5.5	5.5	5.7
uninoculated	6.9	6.9	6.9	6.9	6.9	6.9

standard deviation ±0.02

TABLE 14. Formic acid accumulation in lactose broth after growth of *E. coli* ATCC 11775 and isolate #1840 stock and variant cultures incubated at 35.0, 44.0, and 44.5°C for 48 h, determined by the thiobarbituric acid method.

(tests done in triplicate; averaged results shown below)

	Formic Acid Accumulation (ug formic acid/ml)		
Culture	35.0°C	44.0°C	44.5°C
E. coli ATCC 11775			
stock	525	513	300
var 1	300	338	363
var 2	280	320	363
var 3	325	338	363
var 4	325	338	363
var 5	313	313	343
var 6	263	325	338
var 7	265	320	338
var 8	298	325	325
E. coli #1840			
stock	413	175	13
var 9	350	163	15
var 10	363	163	18
var 11	350	18	13
var 12	388	13	10
var 13	350	15	13
uninoculated	0	0	0

standard deviation ±1

accumulation in lactose broth cultures of the stock *E. coli* ATCC 11775 decreased, while that of the variants of *E. coli* ATCC 11775 increased. Formic acid accumulation in lactose broth cultures of the *E. coli* #1840 stock and variant cultures decreased as the temperature of incubation increased. The decrease was less for the variant cultures than for the stock cultures. Therefore, as the temperature of incubation was increased from 35.0 to 44.5°C, the amount of formic acid accumulated by the *E. coli* variants increased relative to that accumulated by the corresponding stock cultures. This indicated that the conversion of formic acid to gas by the variants was inhibited at 44.5°C, but formic acid was still produced. For the *E. coli* #1840 cultures it was apparent that the formation of formic acid at 44.5°C was reduced, but not lost.

Formic Hydrogen Lyase

Temperature limits for gas production in a formate solution (Durham tube method) using formic hydrogen lyase extracts obtained from 12-h cultures of *E. coli* ATCC 11775 and #1840 stock and variant cultures grown at 35.0°C are shown in Table 15. All enzyme extracts produced gas in a 0.1M formate solution at 43.0 and 44.0°C, but not at 47.0°C. Formic hydrogen lyase extracts of the stock *E. coli* cultures produced small amounts of gas from formate up to 46.0°C, but were unable to do so at higher temperatures. Extracts obtained from the *E. coli* variants produced small amounts of gas from formate at temperatures up to 44.0°C. Five variants

TABLE 15. Gas production in 0.1M formate solution by formic hydrogen lyase extracts of *E. coli* ATCC 11775 and isolate #1840 stock and variant cultures.

(0.1 and 1.0ml enzyme aliquots tested in duplicate; averaged results shown below)

	Gas Production at Each Assay Temperature[1]				
Culture	43.0°C	44.0°C	45.0°C	46.0°C	47.0°C
E. coli ATCC 11775					
stock	+	+	+	w	-
var 1	+	w	ww	-	-
var 2	+	w	ww	-	-
var 3	+	w	-	-	-
var 4	+	w	ww	-	-
var 5	+	w	-	-	-
var 6	+	w	ww	-	-
var 7	+	w	-	-	-
var 8	+	w	-	-	-
E. coli #1840					
stock	+	+	+	w	-
var 9	+	w	ww	-	-
var 10	+	w	-	-	-
var 11	+	w	-	-	-
var 12	+	w	-	-	-
var 13	+	w	-	-	-
uninoculated broth	-	-	-	-	-

[1]Key
ww reactions were very weak for 1.0ml enzyme aliquots, and negative for 0.1 ml enzyme aliquots

(*E. coli* ATCC 11775 var 1, 2, 4, and 6; *E. coli* #1840 var 9) produced gas weakly at 45.0°C when large amounts of enzyme extract (1.0 rather than 0.1ml) were used. These data indicated that the formic hydrogen lyase produced by *E. coli* Lac(g)$^-$ variants was more temperature-sensitive than that produced by the stock cultures. The temperature limit for its activity was slightly extended if a large amount of the enzyme was present.

Acridine Orange Treatment

The stock *E. coli* #1840 cultures treated with acridine orange did not produce any variants for the tests done at elevated temperature. However, acridine orange treatment of the stock *E. coli* ATCC 11775 culture gave rise to some variants. Of the 20 isolates from the 40 and 50 ug acridine orange/ml treatments, 6 and 9 Lac(g)$^-$ variants were detected, respectively. After three subcultures, these variants recovered their ability to produce gas in lactose and EC broths at 44.5°C, but in markedly reduced amounts.

The acridine orange variants that had recovered their ability to produce gas from lactose were retreated with up to 100 ug acridine orange/ml. In this case, only cultures treated with 60 ug or greater acridine orange/ml gave rise to variants for gas production from lactose at 44.5°C. The higher the concentration of acridine orange, the greater the number of subcultures required for recovery of gas production from lactose at 44.5°C.

V. Discussion and Conclusions

This study was undertaken to determine whether phenotypic changes occurred in Enterobacteriaceae, especially the production of gas from lactose and indole from tryptophan by *E. coli* at elevated temperatures, as a result of exposure to treatments that simulate conditions to which organisms are exposed in food handling. The only organisms, besides *E. coli*, that exhibited a change in biochemical reactions tested were *C. freundii* and *S. typhimurium* which showed a weakening, but not a loss, of H_2S production at 44.5°C. For *E. coli* cultures, no phenotypic changes were identified for reactions tested at 35.0°C, but for indole production and gas production from lactose at elevated temperature (44.5°C), stable *E. coli* variants were isolated. As these elevated temperature reactions form the basis of standard methods for detecting fecal *E. coli* in the food industry (ICMSF, 1978), the discovery of such variants is important.

North America uses incubation temperatures between 44.5 and 45.5°C because gas production from lactose at these temperatures is most specific for fecal *E. coli* (Fishbein and Surkiewicz, 1964). In Europe it is believed that specificity for *E. coli* as an indicator organism is obtained by using 44.0°C tests for both indole and gas production (Dufour, 1977). Which conditions are the best for detecting fecal *E. coli* has not yet been decided (ICMSF, 1978). However, for successful detection of *E. coli* under

conditions simliar to those of this study, it would be advisable to use the lower elevated temperature of 44.0°C.

In this study, loss of ability to produce gas from lactose at 44.5°C was more common than the loss of ability to produce indole from tryptophan at 44.5°C. Only three 44.5°C indole variants were isolated, and none of the conditions under which they were obtained (UV, extended storage at 45.0°C, and prolonged cold storage at 4.0°C in TSB) resulted in their repeated production under the test conditions. All variants isolated, including the 44.5°C Ind^- variants, were 44.5°C $Lac(g)^-$ variants. Several of the conditions under which 44.5°C $Lac(g)^-$ variants were obtained (sloppy agar and nutritional study treatments at 4.0 and -16°C) resulted in repeated production of these variants under the test conditions. The relative stability of the Ind^+ trait supported earlier reports that the indole characteristic was more stable than that of lactose fermentation (Anderson and Baird-Parker, 1975; Ewing *et al.*, 1972).

The majority of the 44.5°C variants obtained from *E. coli* ATCC 11775 and isolate #1840 from meat were obtained by random selection or selection of smaller colonies on nutrient agar. Only two variants were found on VRBA plates, as a result of their small size and initial lack of bile precipitation. None of the variants were detected on TBA. However, when 44.5°C Ind^- variants were grown on TBA at 44.5°C, they gave a negative indole reaction. When 44.5°C

Lac(g)$^-$ variants were grown on VRBA at 44.5°C, they formed smaller colonies than the stock cultures. The fact that no Ind$^-$ variants were isolated from TBA and few Lac(g)$^-$ variants were isolated from VRBA might be attributable to the greater selectivity of these media for cells that have been injured. Cell recovery in fact was always lower on TBA and VRBA, than on NA. This agreed with observations of previous researchers, who noted that freeze-injured coliforms are extremely sensitive to solid selective media such as VRBA (Speck and Ray, 1977), and that recovery of injured cells for growth on TBA and VRBA is improved if cells are grown in a pre-enrichment broth prior to plating (Anderson and Baird-Parker, 1975; Stiles *et al.*, 1973). Therefore, the usefulness of TBA and VRBA as media for screening for indole and lactose variants could be limited by the selectivity of these media.

E. coli variants were not detected as a result of repeated exposure to sublethal heat treatment (52.0°C for 15 minutes in TSB). Sublethal heating normally affects the cell membrane, RNA, and DNA (Ordal, 1980), resulting in lower enzyme activities or altered transport kinetics (D'Aoust, 1978). No evidence of such effects was detected in this study, but this damage is seldom mutagenic (Ordal, 1980). Any sublethal heat damage was probably repaired by the cells, so that no change in enzyme functions was observed. Alternatively, the sublethal heating may have injured cells to the extent that their altered or weakened enzyme

functions prevented them from being recovered by the screening process.

Most of the *E. coli* variants were obtained from cultures held for extended periods of time at specific storage temperatures, especially if the medium was low in nutrients, lacked cryoprotective agents, or were broths or semisolid agars, rather than agars. These treatments caused less cell death than either the UV or sublethal heat treatments, which may account in part for the higher incidence of variants detected.

Variants were not obtained from the stock cultures or prolonged storage of the stock cultures on NA slants at 4.0°C, probably because the composition of NA provided an adequate level of nutrients and cryoprotective agents to minimize the effects of cold storage damage (Calcott, 1978; Speck and Ray, 1977). However, variants were obtained after extended storage at 4.0, 35.0, and 45.0°C and cold storage at -16°C in TSB, despite TSB providing cells with a good level of nutrients. This was possibly because the broth would have more water available than the agar, causing adverse freezing and desiccation effects. (Calcott, 1978; Speck and Ray, 1977). Variants were consistently obtained from sloppy agar cultures stored at 4.0 and -16°C and from nutritional studies run in semisolid agars at 4.0°C and in broths at -16°C. The susceptible media were low in nutrients, lacked cryoprotective agents, and had much available water, leaving the cells vulnerable to cold

storage damage (Calcott, 1978; Speck and Ray, 1977).

Variants were not obtained in the nutritional study with media that contained a level of nutrients similar to that of TSB medium and/or included the cryoprotective agent, soytone (Lapage *et al.*, 1970), though variants were obtained repeatedly from nutritional study media which contained a low level of nutrients or lacked soytone. Therefore, it appeared that conditions which failed to protect cells from damage during storage at low temperatures, increased the probability that lactose or indole variants would be produced. Cellular damage produced by prolonged storage is not normally mutagenic (Calcott, 1978) and is often repaired upon resuscitation or subculturing (Ordal, 1970). However, this study indicated that prolonged storage under debilitating conditions could produce 44.5°C Lac(g)$^-$ and Ind$^-$ variants which remained stable upon subculture. This is supported by the discovery of Lac(a,g)$^-$ *E. coli* variants in reactor effluent waters (Kasweck and Fliermans, 1978), and various *E. coli* lactose variants in frozen foods (Mehlman *et al.*, 1974), suggesting that variants in this study may result from prolonged storage. The high rate of Lac(a,g)$^+$ to Lac(a,g)$^-$ conversion observed for *E. coli* in reactor effluent waters (Kasweck and Fliermans, 1978) may demonstrate how the lack of nutrients leaves cells more vulnerable to such damage.

UV irradiation induces DNA damage, resulting in lower or altered enzyme activities if not properly repaired

(Witkin, 1976). Researchers have produced various *E. coli* mutants for lactose fermentation by exposing cultures to different wavelengths of UV light (Lewin, 1977). Based on this knowledge, UV treatment was used in this study to determine if the indole and lactose variants produced by the other treatments examined could be attributed to this type of DNA damage. The UV treatment (daily exposure to 5 minutes of germicidal UV irradiation) produced only one variant. This low incidence was due either to the low sensitivity of the screening technique or the fact that the cells could effectively repair DNA damage. The UV treatment was the most lethal of the treatments used, reducing the viable cell count from 10^9/ml to 10^5/ml, indicating that the few UV variants might be due to the fact that the UV damage was too severe.

All *E. coli* variants retained their original variant characteristics upon subculture. This observation, combined with the knowledge that the treatments to which they were subjected can damage DNA, suggested that the variations were due to genetic damage or alteration, rather than a temporary cellular injury. Though UV irradiation is a well-known cause of such genetic damage (Lewin, 1977), prolonged storage would not normally be considered a cause of genetic damage (Calcott, 1978). The exact reason why permanent alterations in *E. coli* resulted from prolonged storage, especially under conditions of poor nutrition or a lack of cryoprotective agents, was not apparent from this study.

Comparative analyses of the *E. coli* ATCC 11775 and #1840 stock and variant cultures indicated that the variants were most probably derived from their respective stock cultures. Strongest evidence for this was obtained from their identical biochemical reactions at 35.0°C and their antibiotic sensitivity reactions. Additional support was obtained by growth rate analyses for all *E. coli* cultures and serologic analyses for *E. coli* ATCC 11775 cultures. It thus appeared that these variants were actually derived from the respective *E. coli* stock cultures. This suggested that more emphasis should be placed on the elevated temperature test for indole production than gas production from lactose, for the detection of *E. coli* of public health significance. Furthermore, all *E. coli* variants which could not produce indole at 44.5°C also lacked the ability to produce gas from lactose at 44.5°C.

The major pathway for the production of indole from tryptophan by *E. coli* is believed to be the direct breakdown of tryptophan by tryptophanase to indole, ammonia, and pyruvate, with occasional incomplete breakdown stopping at a methyl-indole compound known as skatole (MacFaddin, 1977). The lack of colour development with Kovac's or TBA staining at 44.5°C, indicated that neither indole nor skatole were produced in detectable amounts by these variants (Anderson and Baird-Parker, 1975). Both the activity and synthesis of tryptophanase appeared to be less at 44.5°C than at 35.0°C for the *E. coli* stock and variant cultures tested. This

effect was more pronounced for the 44.5°C Ind$^-$ variants, as shown by the greatly decreased level of tryptophanase synthesis and the lack of tryptophanase activity at 44.5°C. Taylor and Yudkin (1978) attributed similar temperature-sensitive (41°C) *E. coli* mutants for tryptophanase synthesis and activity to mutations in the structural gene (*tna*) for tryptophanase, but did not determine the exact factor responsible for that mutation. The pyruvate assay also failed to indicate conclusively where the variation in indole production occurred. This was possibly due to interference by pyruvate production from other metabolic pathways.

A minor pathway for the production of indole from tryptophan by *E. coli* is mediated by the enzyme tryptophan pyrrolase (Lamanna and Mallette, 1965). The variants exhibited half of the enzyme activity of their respective stock cultures, but no difference in activity was detected between Ind$^+$ and Ind$^-$ variants. Temperature-sensitivity of the enzyme activity could not be tested due to temperature restrictions of the assay (Blake and Kun, 1971). The low levels of tryptophan pyrrolase activity indicated that this enzyme probably does not play an important role in the indole variants detected.

It was concluded that the three 44.5°C Ind$^-$ variants probably failed to produce detectable levels of indole at 44.5°C because of increased temperature-sensitivity of their tryptophanase synthesis and activity. The stability of the

Ind$^-$ characteristic indicated that the variation was probably attributable to damage or alteration of the DNA makeup of the tryptophanase synthesis or encoding pathway. Because of the similarity of these variants to the temperature-sensitive (41°C) *E. coli* variants for tryptophanase synthesis and activity studied by Taylor and Yudkin (1978), it is possible to suggest that the Ind$^-$ variants in this study are also the result of mutation in the structural gene (*tna*) for tryptophanase. However, the exact nature and location of the possible DNA damage or alteration was not determined.

Many *E. coli* variants were found which did not produce detectable levels of gas in Durham tubes in EC or lactose broths at 44.5°C. This trait was only lost by the stock *E. coli* cultures at 47.0°C. The variant and stock cultures grew and produced acid in EC and lactose broths at temperatures up to 48.0°C, so the inability of the variants to produce gas in these media at 44.5°C could not be attributed to differences in growth range. This was important, because poor growth or stricter growth requirements often account for the apparent lack of gas production by fastidious strains of *E. coli* at elevated temperatures (Mehlman and Romero, 1982).

All *E. coli* cultures tested were ONPG$^+$ at 35.0 and 44.5°C, indicating that the 44.5°C Lac(g)$^-$ variants could transport lactose into their cells (Wong *et al.*, 1970) and successfully break it down to glucose and galactose

(MacFaddin, 1977). These cultures were also VP$^-$. Therefore, the lack of gas production by the variants at 44.5°C was not due to an inability to ferment lactose, or a total alteration in the end-products of lactose fermentation, but it was probably due to inhibition within the normal pathway for lactose fermentation.

Most of the gas produced by *E. coli* in lactose fermentation is generated by the conversion of formic acid to CO_2 and H_2 (Bovarnick, 1965; Dockins and McFeters, 1978; Pelczar *et al.*, 1974). The increased formic acid accumulation by the 44.5°C Lac(g)$^-$ *E. coli* ATCC 11775 variants, indicated that the conversion of formic acid to gas at 44.5°C was being inhibited. For the 44.5°C Lac(g)$^-$ *E. coli* #1840 variants, formic acid accumulation decreased, but not to the same extent as the *E. coli* #1840 stock culture, indicating that both formation of formic acid, and the conversion of formic acid to gas by the variant were being inhibited. However, as some formic acid was accumulated by these variants at 44.5°C, they were probably not the same type of variants as those studied by Kanai *et al.* (1975), which failed to produce formic acid at 44.5°C. Therefore, gas production by the 44.5°C Lac(g)$^-$ variants was believed to be inhibited at the level of conversion of formic acid to gas, rather than production of formic acid.

The conversion of formic acid to gas by *E. coli* requires a pH of approximately 6 or less to function optimally (DeLey, 1962), this was not the cause of

inhibition of the breakdown of formic acid to gas by these variants. Conversion of formic acid to gas by *E. coli* also requires a functional formic hydrogen lyase enzyme (DeLey, 1962). Assays using formic hydrogen lyase extracts from *E. coli* cultures grown at 37.0°C, indicated that ability to produce gas in formate solution was lost by the Lac(g)$^-$ variants between 44 and 45°C, and by the stock cultures between 46 and 47°C. It therefore appeared that a more temperature-sensitive formic hydrogen lyase enzyme accounted for the loss of gas production by Lac(g)$^-$ variants at 44.5°C.

Various genes involved in the lactose fermentation pathway of *E. coli* have been shown to be transmissible by F plasmids (Lewin, 1977), hence the possibility that 44.5°C Lac(g)$^-$ variants resulted from of the loss of an F plasmid was studied. The acridine orange treatment produced temporary 44.5°C Lac(g)$^-$ *E. coli* ATCC 11775 variants. The levels of acridine orange used should have eliminated 96% or more of the non-integrated F plasmids originally present (Hirota, 1960). The temporary loss of gas production from lactose at 44.5°C suggested that genes responsible for the encoding of formic hydrogen lyase, or genes bearing indirectly on its function, may have been carried on F plasmids. The few F plasmids which were not cured, or that were protected by integration into the *E. coli* chromosome, would have replicated the genetic data upon subculture, once the acridine orange had been diluted out, leading to the

return of gas production at 44.5°C (Lewin, 1977).

The failure of 44.5°C Lac(g)$^-$ variants of *E. coli* ATCC 11775 and #1840 to produce detectable levels of gas in EC and lactose broths at 44.5°C by the conventional Durham tube method, appeared to be attributable to the increased temperature-sensitivity of their formic hydrogen lyase enzyme. The stability of these variants upon continued subculture and the conditions under which they were obtained suggested that their variation could probably be ascribed to damaged or altered DNA in the genes involved in the synthesis of, or encoding for, formic hydrogen lyase. The exact nature and location of this DNA damage or alteration was not determined, but preliminary studies with acridine orange treatment suggested that the genes involved, or others affecting their function, might possibly be associated with the F plasmid.

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